

Plagiarism, piracy and principles

How honest is the scientific community? Is it, as the story books would have us believe, that every single member of the scientific community is a knight in shining armour, dedicated only to the pursuit of truth and indifferent to where the pursuit will lead? Or may it be, as some would say, that the scientific community is by and large no more honest or dishonest than any other group of professional people in which personal rivalry and ambition cannot be ignored? Might it even be that scientists are if anything less honest, more venial, than the professional groups with which they invite comparison? There is a sense in which these questions and others like them are strictly meaningless. The scientific community is at best a loose aggregation of people with often very different interests, working in laboratories of very different character with techniques that differ profoundly from each other. In some circumstances, scientists work alone, perhaps even in isolation. In others they are members of large teams of people, united by a common interest yet constantly (and understandably) on the look-out to ensure that no one person derives an advantage from the collaboration that cannot be justified by his own contribution to it. There are some who argue that in such circumstances even the notion of a scientific community makes no sense. But there is also a sense in which, from time to time, such questions cannot be avoided. In the past few months, they have been made inescapable by the discovery of cases of widespread plagiarism in the scientific literature, by the latest well-publicized case in which experimental data were as much a product of people's imagination as of laboratory measurement and by the general fear that when times are hard and budgets being reduced in many areas, standards are bound in any case to be at risk.

What is the truth? It is prudent first of all to recognize that recent scandals, however spectacular, are neither as great a slur on the reputation of scientists as many outsiders would like to think nor as novel as many scientists would pretend. There is a tendency among professional scientists reading for the first time tales of large-scale plagiarism (see *Nature*, 12 June and 31 July) to huff and puff, turn red in the face and utter some solemn declaration that something, almost anything, must be done to defend the fair name of science. This tendency should be resisted. It is too often forgotten that the misdemeanours of the present are often mere shadows of the misdemeanours of the past. The seventeenth century, after all, was riven with controversies about whose idea belonged to whom, and people such as Robert Hooke found it sensible to conceal notions such as *ut tensio si vis* in anagrams.

In the days when the importance of data gathered from the real world as the foundation of all theory was not widely appreciated, the theft of ideas pure and simple was easier but also potentially more damaging than at present. In a small part, the conventions by which the scientific profession now regulates itself derive from the bad experiences of these early days. The object of the convention is to give truth an overwhelming advantage over falsehood, originality a powerful edge on repetitiveness and to make it possible that people whose contributions to the stream of discovery are very different are nevertheless able to work easily alongside each other. The scientific community should count itself lucky that these conventions are now so deeply ingrained, so widely respected and so infrequently dishonoured.

It is always something of a tragedy when the conventions are broken, especially if for personal gain or for the sake of personal esteem, but it is also important that when they are found to be broken, the scientific community should not get on its high horse

and over-righteously pursue the transgressors. Especially because the conventions of the profession are so widely known, and because it is so widely understood that the penalty of being found out may be the end of a career, it is only charitable to acknowledge that a deliberate breach of the conventions may be a sign of something pathological, or of insupportable stress. The profession should, in other words, be more compassionate than is often at present the case towards those who fall foul of the conventions and are expelled.

None of this implies that the conventions themselves should be made less strict. On the contrary, there are some respects in which they need to be made tighter. But it is crucial that the scientific community should clearly understand the purposes that the conventions are meant to service. In this connection, even the most primitive of the conventions — that which outlaws falsehood — is not, as some would have it, a matter of morality in some high-minded sense; rather, it has a practical purpose. The scientific community, like any other branch of scholarship, hangs together because of a common interest in a common pool of knowledge. A person who announces the results of experiments showing that water will run uphill, or that chewing-gum becomes superconducting at the temperature of liquid oxygen, or who merely decides to make his way in the world by synthesizing data that confirm some widely held theory, is potentially misleading all his colleagues, wasting their time and diminishing their chance of making a substantial contribution to understanding. Falsehood is thus an impediment to the progress of science as whole, and small falsehoods may have a disproportionately great effect. Whether those who utter falsehoods are immoral in some wider sense is hardly relevant.

But what is a falsehood? The word is not synonymous with the opposite of truth. There is no shame in publishing in the literature an observation which turns out to be incorrect, perhaps because of some instrumental defect or conceptual flaw. Similarly, an entirely wrong-headed theory, the "luminiferous aether", for example, is not an offence against the conventions of the community provided that it is argued on grounds which are explicit, and accessible to all. (From this derives the subconvention that original publications should contain the details needed to repeat an experiment or to reconstruct an argument.) Popper would say that the discovery of untruths of this kind is not merely a chore but the essence of science. Falsehood is different, for it requires of the perpetrator the wish to deceive. Falsehood cannot as easily be rooted out. Telling one from the other without the lapse of time would inevitably entail the impossible task of assessing individual honesty. By convention, it is supposed that everybody is honest — how else could it be? How true is that assumption?

The second primitive convention, common to the whole of scholarship, is that credit must be given where it is due. The objective is not merely that scientists should enjoy that faint glow of pride that follows from discovery or authorship (which is nevertheless a justifiable reward in a profession where other rewards are few and far between) but that it should be possible for others to reconstruct the origins of new ideas, putting them in a wider intellectual context and making them intelligible. But failure to acknowledge sources adequately and generously is also bound to cause personal offence — people are human, after all — which can seriously damage the free exchange of data and ideas.

These, as they must be, are high ideals. How well are they kept? The recent flurry of plagiarism is not a good guide to present



standards, if only because plagiarism is so easily detected and demonstrated. Plagiarists are either fools or desperate people. Concocting false data, cooking the books, is a more private matter and therefore harder to detect. Many heads of busy laboratories will be alarmed to think what may be happening without their knowledge — and will no doubt come to the conclusion that their only safeguard is to be directly involved in their own research, not merely as leaders but as colleagues of those who do the work. Even so, clearcut transgressions are likely to be rare. Less flagrant misdemeanours, or complaints thereof, are much more common. A helpful referee may find that his constructive comments become the gist of somebody else's publication. A member of a coordinated research programme may find that the contributions he has made to the planning of an enterprise turn up in the literature without his knowledge. Or references to important contributions to some field may be skimmed for the sake of a false impression of originality. There is a lot of this about.

Remedies are unfortunately not easily devised. In the old days (half a century ago) it was the common practice for the head of a

laboratory to take responsibility for everything that happened in it or was published from it. Grand old men used to terrorize their juniors yet give their work the stamp of authenticity. To turn back the clock would be impossible and would solve nothing, for even grand old men are capable of falsehood. Yet there is something in the view that the standards of behaviour would be improved if only laboratories as units engaged more freely and openly in discussions of their collective work. Falsehood is necessarily a private matter; open discussion must help to show it up — or even show that falsehood is unnecessary. It is only proper to record that many of the best laboratories are among the most willing to talk domestically. It would also help if external pressures on individual scientists could be abated. The competition for grants, promotion and kudos is the driving force. The need to publish derives from that. There is at least a chance that these pressures would abate substantially if the methods of financing research were only subtly changed. Shifting the balance of decision from external agencies to laboratories themselves would be certain to help. Is this the constructive course to follow after the disclosures of the past few weeks?

Pesticides in developing countries

Many years have passed since the argument about the future of the environment in which people live was sharply polarized, dividing those who considered that the surface of the Earth is a robust place and those who argued that, on the contrary, quite small changes affecting the ecological balance might have catastrophic consequences. Mercifully, these discussions are now conducted more moderately. Both sides in the old argument seem to have decided that everybody's interest will be served if consensus rather than conflict can be built around environmental problems. Unfortunately, however, it seems that old habits die hard. From time to time, vestiges of the sharp divisions of the 1960s crop up in public statements and in the newspapers. One of the citadels of the extreme environmentalists appears to be the journal called *New Internationalist*, published by no less an organization than Oxfam, widely respected for the way in which it has tackled problems in developing countries with energy, compassion and imagination which are rare even for the most dedicated voluntary organizations.

New Internationalist is, however, over-imaginative in the way it tackles problems of the environment in developing countries. Thus, in the July 1980 issue of the journal, an article by the editorial staff of the journal which appears under the heading "Peddling pesticide" begins with the declaration that "until DDT was banned as a proven cancer-causing agent it was the penicillin of pesticides . . .". The article goes on to argue that the chemical companies, disconcerted to have been "caught pushing poisons", stopped manufacturing DDT in developed countries but set about selling their manufactured stocks in countries overseas. The objective of the article is to argue that the multinational chemical companies are using the developing world as an outlet for sales that would otherwise be frustrated and asks that Western governments should put a stop to their wicked ways. The argument thus brings together two important elements of the demonology of the extreme environmental movement of a decade ago — big business in general, and international business in particular, and pesticides in general, DDT in particular. Nobody quite understands where these demons have sprung from but no doubt they have their roots in a variety of improper practices in the past. For there is no doubt that at many periods in the past century developing countries have been exploited unfairly by business interests from overseas. Similarly, it is beyond dispute that DDT and the other organochlorine pesticides were used indiscriminately and perhaps irresponsibly soon after their introduction — although it appears that most of the damage that followed was in North America, where agriculture is intensive,

and not in the developing countries. None of this, however, excuses what *New Internationalist* has to say about DDT, especially because what the developing countries need to know most urgently is more complicated.

The truth is that DDT is not a poison in any reasonable meaning of that word. Its acute toxicity to people is low, which is why it was widely used, a quarter of a century ago, for the control of mosquito populations in the areas of the world where malaria is endemic. Similarly, the evidence that DDT is "cancer-causing" is ambiguous in the ordinary meaning of those words. It is true that high doses of DDT fed to laboratory animals will induce cancer, as indeed they provoke disturbances of liver function. But there is no evidence that the use of DDT has in the past caused damage to people when used with suitable precautions and in circumstances that are appropriate. The more toxic organochlorine pesticides are more damaging to animal life, but even they have not been taken off the market in most industrialized countries because of the damage they might do to people — rather they have been controlled and sometimes banned for fear of what they might do to wildlife populations.

In developing countries, circumstances are different. It may be regrettable but it is true that many governments in developing countries are less squeamish about wildlife populations than are the governments (and the electorates) of industrialized countries. *New Internationalist* would be fighting a good cause if it sought to alert them to the importance of a balanced wildlife population and to the importance of refraining from needlessly disturbing the natural ecology. In due course, many such governments would no doubt follow industrialized states in controlling DDT for fear of unwanted effects on the wildlife population. But for many of these governments there is a more urgent reason why powerful pesticides such as DDT should be used with discrimination. The original enthusiasm for the use of DDT in controlling malaria has evaporated now that problems of insecticide resistance have become natural restraints on what can be accomplished. Similarly, in the industrialized countries which practise intensive agriculture, the United States and Western Europe in particular, there are growing dangers that the uncontrolled use of pesticides may serve chiefly to stimulate the emergence of resistant insects. The difficulty is that such problems cannot be solved simply by taking one, or even several, offending chemicals off the market — somehow, instead, their use has to be planned imaginatively. Is not this the point that *New Internationalist* and its parent organization should be seeking to establish?

Uranium exposure limits in dispute

A new dispute has arisen between epidemiologists and health physicists over the interpretation of data on the health effects of low levels of ionizing radiation, the subject of a recent report from the National Academy of Sciences (*Nature* 7 August p. 550).

This time the disagreement centres on whether current exposure limits to the radioactive gas radon and its decay products may present an unnecessarily high risk of lung cancer to the 7,000 uranium miners currently employed in the United States.

And the precise shape of the low-level dose-response curve has been given a new significance by growing concern about the possible health risk to the general public from radon-emitting building materials, since radon's precursor, radium-226, is a trace element contained in most rocks and soil.

In the light of epidemiological evidence on occupational exposures that has been accumulated since the current standards were set in 1971, the National Institute of Occupational Safety and Health (NIOSH) has suggested to the Department of Labor that permitted exposure limits for uranium miners should be significantly reduced.

However, the International Council for Radiological Protection (ICRP) argues that no change is needed. In a set of new recommendations currently being circulated to its members, the ICRP says that on the basis of dosimetric techniques applied to models of the lung, the current exposure limits appear to be adequate.

Mining companies — already facing a tight economic squeeze because of a virtual collapse in the international market for uranium ore — are using the ICRP's arguments to press the department not to reduce the limits. A move to reduce the limits could mean that mining companies would have to install expensive ventilation equipment.

Research in the 1950s and 1960s confirmed an abnormally high incidence of lung cancer among uranium miners. The cause was identified as radon and its alpha-emitting decay products — known as radon daughters — which become attached to air particles and can lodge in miners' lungs. The Department of the Interior introduced in 1971 an exposure limit of 4 working level months (WLM) a year, with a ceiling airborne concentration not to exceed 1.0 WL. (One working level (WL) is defined as a radon daughter concentration in one litre of air resulting in the emission of 1.3×10^5 MeV of potential alpha energy).

Since then, evidence from epidemiological studies in Canada, Sweden, Czechoslovakia and the United States, suggests, according to a report put together by a NIOSH study group, that the full effects of radon exposure may have been

underestimated, and that "there appears to be no margin of safety associated with the present standard".

The working group point out that triggering doses may be substantially lower than the cumulative doses associated with a particular incidence of lung cancer, since the radiation absorbed after the triggering dose may have been biologically redundant. In addition, the contribution of smoking to lung cancer among miners may have been overestimated in previous work. Some studies appear to show a higher rate of lung cancer among miners who do not smoke than among those who do — perhaps because cigarette smoking creates a thicker layer of mucus which may provide some protection against alpha emissions.

In submitting the report to the Department of Labor's Mine Safety and Health Administration (MSHA) Dr Anthony Robbins, Director of NIOSH, says that the apparent excess risk of lung cancer among those who might accumulate 120 WLM over a working lifetime — perhaps twice previous estimates — was a cause for major public health concern.

"On the basis of the study group's report, we feel that the present MSHA standard of 4 WLM per year does not provide an adequate degree of protection for underground miners exposed to radiation when it is evaluated over their exposure lifetime," Dr Robbins says.

NIOSH officials met with MSHA at the beginning of this month, and agreed that there is enough scientific evidence to support a possible revision of the regulation.

The department has already been petitioned to reduce permitted exposure levels by Ralph Nader's Health Resources Group and the Oil Chemical and Atomic Workers, a labour union to which many of the uranium miners belong. The two organizations are demanding that the maximum allowed radiation be reduced from 4 to 0.7 WLM per year.

However, the department is being urged by the industry to take into account the ICRP regulations suggesting little need for change. The ICRP proposals, agreed in draft form at a meeting of the commission in Brighton, UK earlier this year, describe two different ways of estimating what might be considered a safe exposure level.

Using epidemiological techniques based essentially on the same data as those analysed by the NIOSH working group, ICRP suggest that a safe limit would be between 2 and 10 WLM per year.

However, the ICRP also makes estimates based on a dosimetric analysis of the expected effect on the lung of a given amount of radiation, and in particular to that region of the lung particularly susceptible to alpha emission.

The latter approach, according to the ICRP, leads to a recommended exposure limit of 5–8 WLM per year based on a regular lung dose, or 9–14 WLM per year based on a mean lung dose. And given the variability in the epidemiological data, ICRP suggests that a figure of 5 WLM should be used as the basis for regulation — adding that for uranium miners already exposed to additional gamma radiation, this limit would tend to be reduced by 20 per cent for radon daughters alone, bringing it in line with the current US standard.

Ironically this conclusion is somewhat in conflict with the judgement of the National Academy of Sciences' Committee on the Biological Effects of Ionizing Radiation (BEIR), which last month moved closer to ICRP's position that for a different type of low level radiation (that resulting from X rays and gamma rays) a linear dose-response model may overstate the dangers.

In its comments on the dangers from alpha emission and other sources of high-LET (linear energy transfer) radiation, however, the BEIR committee drew heavily on the epidemiological studies of uranium miners and others to conclude that "the application of the linear hypothesis is less likely to lead to overestimates of risk and may, in fact, lead to underestimates".

David Dickson

High-altitude research

Reflated hopes

A threat to halt the launching of large scientific balloons from two sites in Australia may yet be averted if a proposal to update equipment and share running costs is accepted by the five countries which regularly use them. The continuation of the facilities is particularly important for astronomers from Australia, the United States, Britain, Germany and Japan who want to send balloon-borne payloads to high altitudes to study X-ray and UV sources in the Southern Hemisphere near the Galactic Centre.

The Australian government has threatened to close the facilities at Mildura, Victoria and Alice Springs in the Northern Territory because it says that it is heavily subsidizing foreign astronomers whose governments do not pay an economic fee. The United States is the only country that currently contributes to running costs: it pays a third, Australia making up the remaining two thirds. Other users of the facility currently pay about £10,000–11,000 for a launch and nothing towards basic running costs.

Britain, Germany and Japan have each been launching on average two to three balloons per year. Australia started searching for alternative funds four years ago when Britain was the main foreign user apart from the United States. An attempt then to get Britain to pay one third of the running costs, about £50,000, failed with Britain's Science Research Council pleading poverty.

The current threat to close the facilities unless outside funds are found quickly seems likely to produce results. Britain and the United States have agreed to accept a proposal, put by Professor Vic Hopper of Melbourne University during recent visits to both countries, to share running costs between the five principal users and to update some of the out-dated equipment.

The new proposal appeals to Britain in particular because the cost would be shared by more countries, Germany and Japan having become regular users of the facilities over the past four years. The plan would also allow user countries to up-date equipment by providing their own. Hence the United States and Germany might consider providing a better launch truck for heavier balloons and Britain would be willing to provide an improved kilobit telemetry link from its now abandoned Skylark rocket programme. Professor Hopper left for Germany and Japan last week to discuss his ideas there.

Britain seems to have come round to the view that it can hardly refuse Professor Hopper's proposal, especially if the United States, Germany and Japan also agree to cooperate. Compared to the cost of other collaborative projects with Australia, such as the Anglo-Australian telescope, the cost would be almost insignificant. In the interests of international collaboration, at least, it would probably be worth it.

Judy Redfearn

Nuclear reactors

Czech closure

In a sudden policy about-face, the Czechoslovak Nuclear Energy Commission has admitted that the country's first nuclear power station at Jaslovské Bohunice had to be closed down permanently after a major incident a few years ago. Speaking on Prague Radio, Miloš Drahný, a senior official of the Commission admitted that a serious defect had damaged the 440-MW No. 1 generating set at the country's first VVER-440 light-water nuclear power station.

This admission follows almost two years of semi-official denials backed up by glowing accounts in the media of the benefits which the station was bringing to the Czechoslovak national economy (a claimed 2 million MW generated in 1979). As recently as 17 July 1980, Prague TV carried a news item reporting the completion of "summer maintenance work" on the No. 1 set.

The closure of the station was first reported in November 1978 in Document 22 of the Charter 77 movement. This document reported two accidents. The first, on 5 January 1976, was due to an obstruction in the safety valve of a fuel cell (probably due to human error), leading to an escape of radioactive coolant (CO_2) and the death of two workers who could not be evacuated in time, since one of the emergency exits had been locked as part of an anti-pilfering campaign. The second accident, on 24 February 1977, was due to human error in the loading of a fuel cell, producing a rupture in the primary circuit and extensive contamination in the primary and secondary circuits. Activated steam was voided into the atmosphere, and there was an escape of liquid into a nearby stream, which had to be fenced off temporarily.

The public discussion which the Chartists had hoped to evoke never materialized. The authorities took the stance that there was no accident. But the total absence of sound data (workers at the plant who had been at risk in the first accident were not even allowed to know the extra dosage they had absorbed) caused rumours to proliferate. By 1979, it was widely believed that the liquid spilled from the second accident had got as far as the Danube — an allegation which the Chartists themselves have never either supported or denied, and which on the face of it would be very difficult to substantiate since the Danube Commission deals only with pollution caused by ships and there is, to date, no overall monitoring system for the river.

Document 22 in its concluding paragraphs called for a moratorium in the Czechoslovak nuclear energy programme until the public had been informed of, and had had a chance to consider, the hazards involved. But with the authorities firmly committed, like all Comecon countries, to nuclear power, construction went on apace; the No. 2 set at Jaslovské Bohunice is now undergoing acceptance trials, the Dukovany station is under construction and work on the Mochovce station has been started.

Nevertheless, and without any reference to Document 22, the Czech and Slovak media have from time to time appeared to be answering the Chartists on specific issues. One of the main criticisms made in Document 22 was of the psychological and physical health of the staff at Jaslovské Bohunice. The majority of workers, said the Chartists, were willing to work dangerously long hours without proper safety precautions in return for high pay and consoling themselves with alcohol and petty pilfering. Official releases countered this with descriptions of health and safety regulations, and the careful psychological monitoring of prospective employees. Other articles stressed safety precautions and contingency plans for antipollution measures in the event of any serious

radiation leaks.

Drahný's belated announcement that such an accident has, in fact, already occurred, will come as no great surprise to the Czechs and Slovaks, accustomed as they are to the rewriting of the past. They may be intrigued, however, by the timing of the announcement, which came a few days before the anniversary of the 1968 Soviet intervention.

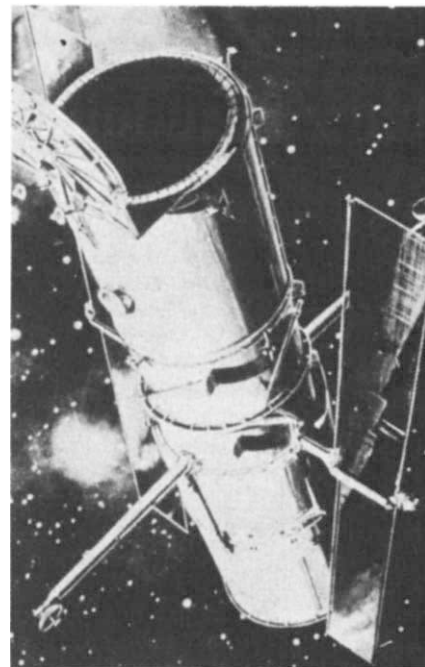
Vera Rich

Space telescope

Edinburgh's bid

Britain's Science Research Council (SRC) is to put in a bid to the European Space Agency (ESA) to house the European space telescope coordinating facility at the Royal Observatory, Edinburgh. Bids are also expected from the European Southern Observatory at Garching, West Germany, at least one astronomical institute in France and possibly one each in Spain and Italy. ESA plans to decide on a site early next year.

European scientists will receive at least 15 per cent of observing time on the space telescope — a joint ESA-US National Aeronautics and Space Administration (NASA) project — due for launch aboard the space shuttle in late 1983, roughly in proportion to ESA's contribution to its capital cost. The task of the European Coordinating Facility (ECF) will be to receive data from NASA's Space Telescope Science Institute, and to analyse and disseminate them to European users of the space telescope, within a matter of days of receipt of the data in the United States. But bigger plans are afoot for the ECF. It will be designed to handle all data produced by the space telescope; ESA is currently negotiating with NASA to receive data



The space telescope — as it will look

which are not initially allocated to European astronomers when exclusive rights to it have expired after one year.

The SRC believes that its proposal stands a good chance of success because it would use Britain's Starlink facility which already employs highly sophisticated methods of image processing to analyse data from ground-based telescopes. The Royal Observatory in Edinburgh houses one of Starlink's six nodes. European astronomers would be able to access space telescope data quickly by travelling to Edinburgh or only slightly more slowly at one of the sites of the five other nodes, three of which are within easy reach of London, the other two being in Manchester and Cambridge. One of the main reasons for choosing Edinburgh rather than any of the five other institutes housing a node is that from next October, Dr Malcolm Longair will be director of the Edinburgh observatory. He has been closely involved in the space telescope programme since its inception.

To cope with the enormous increase in data, however, facilities at the Royal Observatory would need to be expanded, particularly by enhancing the existing image processing unit and building a special unit to cope with the data.

ESA plans to pay about £66,000 towards the capital cost of the facility, about £33,000 per year towards running costs and the salaries of seven staff. The host institution will be expected to find the rest of the cost from its own pocket.

The SRC estimates that to build the facility at Edinburgh, it would have to provide about £250,000 and pay for an extra five staff.

Judy Redfearn

German workforce

Ten years on

Microcircuits and other new technologies likely to be adopted in the 1980s in West Germany will result in the loss of 1.8 million jobs in some low-skill sectors, and a gain of 1.9 million in other higher-skill occupations, says a new forecast commissioned by three Federal German ministries.

This massive shift in employment will require government policy "specifically shaped to counteract what will be an increasing tendency towards conflict in the community" says the report, prepared by Mackintosh Consultants Ltd of the UK and Prognos AG of Basle.

The German government has described the report as a brilliant study and awarded the teams a prize for their efforts. Mackintosh Consultants reviewed probable technical developments by making studies in the UK, Japan, Germany and the USA; and Prognos AG looked at social and economic factors, including union and management attitudes affecting their

Economically active persons classified by type of activity in FRG for 1977 and 1990

Field of activity	Economically active persons		Change: 1990 compared to 1977 (%)
	(thousands)		
	1977	1990	
1 Mainly management functions	922	1,104	19.7
2 Office work	3,863	3,611	-6.5
3 Entertainment, publishing	125	152	21.6
4 Education	897	1,076	20.0
5 Cleaning operations	872	1,002	14.9
6 Installation, repair and maintenance of machinery	2,224	2,695	21.2
7 Specialized consulting, R&D	1,171	1,604	37.0
8 General supply services	2,343	2,150	-8.2
9 Special supply services	498	470	-5.6
10 Assemblers and assistants	2,467	2,144	-13.1
11 Rail transport	199	165	-17.1
12 Road and sea transport	748	797	6.6
13 Operating processing plants	1,396	1,000	-28.4
14 Special tasks in manufacture of metal products	780	801	2.7
15 Special tasks in construction	1,371	1,095	-20.1
16 Special tasks in food, beverages, tobacco	501	531	6.0
17 Special tasks in agriculture	1,610	1,372	-14.8
18 Special tasks in mining	151	132	-12.6
19 Special tasks in manufacture of clothing	200	200	0
20 Special tasks in printing	151	104	-31.1
21 Transmission of information	176	157	-10.8
22 Health services	727	917	26.1
23 Legal advice, business consultancy	126	158	25.4
24 Security functions	927	1,120	20.8
25 Test, measurement	376	371	-1.3
26 Others	100	64	-36.0
Total	24,921	24,992	0.3

introduction in the current decade. Both studies were detailed and sectional, dividing the economy into at least 50 sections and jobs into 26 categories, all of which were looked at individually (see table).

The study identified 15 sensitive sectors of the German economy where the pressure to innovate was great, but the ability to respond small. Prominent among them were the chemical industry and refineries, where the price of imported oil will require conversion to new feedstocks; but conversion will require massive investment in new capital equipment, new skills in the workforce and possibly new locations for industry to meet the distribution requirements of new raw materials.

Jobs directly connected with the introduction of the new technologies — such as installation, and the repair and maintenance of what may initially be unreliable (because untested) technologies — would increase during the 1980s by 471,000. But typically the qualifications required in the new jobs will be far in excess of the jobs being eliminated: spotting if a cherry is on a cake requires less education than spotting the fault in the device which detects cherries on cakes.

The area in which most jobs would be lost is in the operation of processing plants, largely through the introduction of micro-processors to handle decision-making on production lines. Here, the report estimates, there will be 396,000 fewer jobs in 1990 than there were in 1977. "Assembly", categorized separately in the study, would also lose 323,000 jobs, through reductions in numbers of components and the automatic assembly of, for example, television sets.

The Bundesministerium für Forschung und Technologie (BMFT) have estimated

the range of unemployment which might follow from the Mackintosh-Prognos scenario, given different conditions of economic growth and government and industrial investment. At a pessimistic level of 2 per cent annual growth in gross national product (compared to the Mackintosh-Prognos estimate of 2.9 per cent), investment would be expected to be low, and unemployment would rise from 1.03 million this year to 2.23 million in 1985 and 2.4 million in 1990. It would fall again to 1.44 million in 1995, the BMFT estimate.

Mackintosh's project director for the study, Mr Tom Jacobs, said last week the likely speed of innovation would be great in the next decade but would need great social change. Hence, says the report, Germany must develop "a high level of social communication and willingness to support common objectives, which is only possible if based upon a high level of overall understanding of where the community's best interests lie. Thus, the management of social learning processes becomes a central task in politics."

Robert Walgate

Environmental carcinogens

Nitrite safe?

The US processed-meat industry received a welcome reprieve last week when the Food and Drug Administration (FDA) and the Department of Agriculture (USDA) announced that there was "insufficient evidence" to label sodium nitrite — a widely used meat preservative — a carcinogen.

Two years ago results of experiments contracted by the FDA to Dr Paul M. Newberne, Professor of Nutrition and Food Sciences at the Massachusetts

Institute of Technology (MIT) suggested that nitrite produced cancer in the lymphatic system of laboratory rats.

Almost 13 per cent of 1,350 test animals in the study were said to have developed lymphomas, compared with only 5.75 per cent of the 573 not being fed nitrites.

The FDA had proposed a phased withdrawal of nitrites, but at the same time had announced that the MIT study was to be placed in the public record "so that the process of external scientific scrutiny can commence". As well as gathering comments from their own scientists, the FDA and the USDA commissioned a Washington-based contract research group, Universities Associated for Research and Education in Pathology, to review the 50,000 tissue slides taken by Dr Newberne of his 2,000 test animals.

Last week the two agencies announced that the review of the slides revealed insufficient evidence to support Dr Newberne's conclusions that nitrites increased the risk of cancer in the lymphatic system. The review group said that it found fewer of the slides indicated the presence of cancer than Dr Newberne had concluded, and the agency scientists agreed.

Some of the supposed lymphomas were noncancerous, the group said, and some were histiocytic sarcomas, which have no known human counterpart.

On the basis of this reassessment of Dr Newberne's data, the two agencies announced that they were dropping plans to phase out the use of nitrites.

However, the statement emphasized that steps are still being taken to reduce the level of nitrites in food because of concern that, through the effects of cooking or digestion with other chemicals, nitrites can form nitrosamines, known potent carcinogens.

Following last week's announcement, the FDA has also asked the National Academy of Sciences to review and evaluate all available scientific data about nitrites, and to analyse possible alternative preservatives for use in the meat and poultry industries.

The decision not to impose a ban on nitrites was widely welcomed by food industry officials, where nitrites are used not only to prevent the spread of botulism, but also to give meat the red colouring favoured by US consumers. The industry had complained that there was no alternative agent with equivalent taste, texture and handling characteristics, and had previously claimed that a ban might destroy the US pig industry, over 70 per cent of whose products are processed with the use of nitrites.

When his research results were reported two years ago, Dr Newberne urged that they should be confirmed by other research workers before any ban was introduced. Last week he was on vacation, unable to comment on the review group's critique of his research techniques.

David Dickson

Energy conservation

UK forecasts

The UK Atomic Energy Authority (UKAEA) has dismissed as "uneconomic and risky" proposals for an aggressive policy on energy conservation put forward by the International Institute for Environment and Development (IIED) 15 months ago.

That "low energy strategy" placed too much emphasis on conservation and too little on the expansion of indigenous fuel supplies say the UKAEA. Furthermore, the strategy leads to a shortfall in oil and gas supplies of up to 108 million tonnes of coal equivalent in the year 2025, say the authority, overestimates the savings possible in the domestic sector, and is inconsistent in its projections of sectional and overall economic growth.

What no doubt stuck in the authority's throat was the claim in the IIED report that electricity output can be met by building only 6 GW of nuclear capacity in the first quarter of next century, so that nuclear power would become "a peripheral issue", and that the fast breeder "could be shelved indefinitely". (In contrast, the UK government has since announced a 15-GW nuclear construction programme over the decade beginning 1982.) Thus the authority wish to argue that energy demand, and particularly electricity demand, will remain more buoyant than the IIED calculate.

Gerald Leach, principal author of the IIED report, last week admitted that the low energy strategy could lead to an oil and gas shortfall in 2025, in so far as it was possible to calculate that distance into the future, but said that it would be very easy to make good the shortfall by, for example, increased coal production for which his estimate for the year 2025 had been more conservative even than the 170 million tonne target set for the year 2000 by the National Coal Board.

In domestic conservation, the level of savings depended greatly on the assumed price ratio between gas and electricity as a function of time, said Leach. "Everyone is crashing out of electricity now", and electricity prices will increase further as coal and oil become more expensive. So if cheap nuclear electricity does come on line in the UK in the 1990s it may be too late.

On the other hand, argue the UKAEA, the assumption in the low energy strategy that real incomes in 2025 will be 3 times those of today, and that electricity prices relative to gas will be 2.4 times cheaper than today, are incompatible with the assumption that domestic electricity use will decline. The greater heat gains available from electric heat pumps and their quietness, compared with gas-driven heat pumps, will lead to their preferential adoption, say the authority; and the IIED have ignored a likely trend to greater size in domestic appliances. UK refrigerators

average 5-6 cubic ft, whereas American ones average 12 cubic ft.

But potentially the most damning criticism in the UKAEA reply is that the IIED report is inconsistent. One of its most attractive conclusions was that high growth was possible alongside a reduction in energy use; but the assumed growth rate is not matched by the sectional estimates from which energy use is derived.

The IIED report, in its high growth case, assumes 2.94% annual growth in gross domestic product and the growth of non-energy manufacturing industry at only 2.19%. But that implies, the UKAEA point out, that the other sectors (which are mainly service industries) grow faster than 2.94%, whereas the IIED figures show much lower growth in the measures used (for example, floor area for the commercial and institutional sector).

Leach's reply to this is that he assumes, on the basis of many interviews and studies, that the monetary productivity of a given unit in the service sector will increase. For example, the £/m² generated in office work will increase through the introduction of microcircuitry.

Robert Walgate

Soviet space

Salyut women

Although Soviet planners decided in 1976 not to recruit any more women to follow Valentina Tereshkova into space as a pilot, they also announced that women would still be eligible to serve aboard space stations as doctors, astronomers or stewardesses. None has yet done so but it seems as if a welcome is now being prepared aboard Salyut.

Until now, the Salyut stations have been somewhat cramped, and, according to an unattributed talk on Moscow radio last month, "quite hard and difficult even for men". Now, however, Salyut-type stations have "more acceptable" conditions for joint work by both men and women. In the near future, stations will have separate cabins, rest rooms and showers.

Salyut 6 already has an experimental shower cabinet, in which the cosmonaut is sluiced by a stream of water drops borne by an airflow. In the future the beads of water which remain in the shower cabinet instead of passing on to the recycling system will be dealt with by the newest aid to space hygiene, a special towelling drying-bag.

One on-board luxury the would-be citizeness-cosmonaut still cannot expect is the traditional bunch of flowers on Women's Day (8 March). Soviet space biologists announced last month that they were abandoning attempts to grow flowers in space, for lack of positive results, and they were now concentrating their attention on radishes, cucumbers and carrots, which could form an important addition to the cosmonauts' diet.

Vera Rich

NEWS AND VIEWS

Lamarckist revival in immunology

From R.B. Taylor

DARWIN'S concept of natural selection has gained almost universal acceptance. Along with it most biologists also tacitly accept Weissmann's doctrine of the inviolability of the germ-plasm, and so reject the possibility that acquired characteristics could be inherited. The old controversy on this last point has recently been re-opened by E. J. Steele. In his book (*Somatic selection and adaptive evolution*, Williams-Wallace, Toronto-London, 1979) he assembles evidence that acquired characteristics may be inherited, and suggests how this might occur. His proposal is based on Temin's provirus and provirus hypotheses for the evolutionary origin and biological role of the C-type RNA viruses (*J. Nat. Cancer Inst.* **46**; *Science* **192**, 1975). These viruses can transduce genetic information from one somatic cell to another by capturing intracellular RNA sequences (e.g. messenger RNA) and infecting other cells where the RNA is copied by reverse transcriptase into DNA capable of being integrated into the genome. It would require only that such information be transduced into germ-cell DNA for inheritance of acquired characteristics to become conceivable — as indeed was suggested by Temin. If somatic mutations are capable of being selected by environmental pressures (as is widely accepted for the lymphocytes of the immune system) then this would increase the probability of their being transmitted to the germ-line and fixed as heritable characteristics.

This neo-Lamarckist hypothesis has recently received experimental support in a paper by Gorczynski and Steele, (*Proc. Nat. Acad. Sci. U.S.A.* **77**, 2871; 1980) who make the startling claim that neonatally induced immunological tolerance to MHC (major histocompatibility, or transplantation) antigens can be genetically transmitted to the offspring through at least two generations.

The experiment consisted first in the induction of tolerance in male mice of the CBA strain by the classical procedure of neonatal injection of lymphoid and bone marrow cells from a hybrid donor strain bearing foreign MHC antigens: (CBA × A)_F₁ (Billingham *et al.* *Phil. Trans. R. Soc. B* **239**, 357; 1956). The neonatal injection was followed by regular

further injections: a procedure known to induce a state of chimaerism. When they reached adulthood these tolerant males were mated with normal females. Some of the offspring from this first mating were killed and examined for tolerance by testing the ability of their spleen cells to mount a cytotoxic (CTL) response *in vitro* against target cells of the donor strain. Others were allowed to survive and were mated either with each other, or with normal CBA mice to give a second generation. These, and their first generation parents, were likewise examined for tolerance by CTL assay. Both generations, neither of which had been intentionally exposed to the tolerogen, showed a wide range of response, from normal down to undetectable. The important point is that a high proportion of them (50–60% of the 1st generation and 20–40% of the 2nd generation) gave responses significantly below those given by control CBA mice. Specificity was demonstrated by the fact that the mice responded normally to a 3rd party strain: C57B1/6J.

Such a remarkable result must be questioned very closely — both as to its technical adequacy, and as to the interpretation given to it. The CTL assay used in the experiment is a standard procedure and does not demand any major technical innovations. Although most of the results depend on one-point assays, the same degree of hyporesponsiveness was seen even at a fourfold higher concentration of attacker cells. One might ask for more controls to show that the inherited patterns of response were really dependent on the initial induction of tolerance, and not on a tendency of some CBA males to throw hyporesponsive litters. One might also ask that the tests *in vitro* should be supported by evidence of graft tolerance *in vivo*.

The further interpretation leaves even more room for questions. We must ask (a) *what*, in terms of phenotype, was transmitted; and (b) *how* was it transmitted? To answer the first question we need to define immunological tolerance in its various guises, and ask to which of

these (if any) the Gorczynski-Steele phenomenon belongs.

In its broadest operational sense tolerance means a state of specific unresponsiveness, which includes states of significant hyporesponsiveness such as were found in many of the mice claimed to have inherited tolerance. It can exist in T cells or B cells or both. Underlying the condition of tolerance may be any of a variety of mechanisms which fall into two categories: those in which active suppression by suppressor lymphocytes has been demonstrated, and those in which it has not, and which are assumed therefore to be due to irreversible 'deletion' of competent cells. This distinction is important because although somatic selection could act to favour a mutant with positive function (suppression) it could not act on a loss mutant. The usual test for distinguishing between deletion and suppression is to make a cell mixture in order to see if the tolerant cells will be able to prevent the normal cells from responding. However, this test could not necessarily be used to establish that suppressive mechanisms are operating in the mice with inherited tolerance. This is because it would not detect suppressor cells whose action was to *prevent the development* of competent cells from stem cells. It is this kind of cell which has recently been detected (by a different test) in mice which would otherwise be thought to have a deletion-type of tolerance (Gorczynski and MacRae *J. Immunol* **122**, 737 & 747; 1979).

The agents of active suppression have been identified both as suppressor T cells and as free antibodies. In both cases, there is now evidence that they may carry specificity for the variable regions (idiotypes) on the antigen receptors of the cells they suppress. The situation becomes even more complex when we consider artificially-induced tolerance to MHC antigens, because the tolerance-inducing inoculum usually contains cells capable of reacting against the host. This they can do either by the unexpected, but now well established ability of F₁ hybrid cells to recognise parental strain MHC antigens (Shearer, *et al.*, *Cold Spring Harbour Symp.* **41**, 511; 1976); or by means of an anti-idiotypic response against the

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receptors by which the parent recognises the foreign antigens in the F_1 (Ramseier & Lindenmann *Pathol. Microbiol.* **34**, 379; 1969). This latter mechanism can suppress the parental cells so as to produce what amounts to a phenotypic state of tolerance (Binz *et al.* *Nature* **246**, 146; 1973). Thus a state of tolerance can involve any of a wide variety of different mechanisms, all of which may represent facets of the way self-tolerance is maintained. At least some of the positive suppressor mechanisms however seem to be specially stimulated after induction of tolerance by artificial means. This is important because it is clear that natural self-tolerance is not inherited; if it were one would expect to see it in the homozygous offspring of an $F_1 \times$ parent mating. In fact however such mice do not tolerate the foreign F_1 antigens any better than their homozygous, non-tolerant parent.

It is possible however that Gerczynski's and Steele's mice have inherited, not their fathers' suppressive mechanisms, but the antigen to which the fathers have been made tolerant. Very large numbers of donor cells were injected at regular intervals throughout the fathers' lives, so genes specifying donor antigens could as easily be imagined to have found their way into the germ-line as somatically mutated paternal genes. In that case such genes would be expressed in a proportion of the offspring and would be recognised and tolerated as 'self'. This possibility is mentioned by Gerczynski and Steele.

This brings us to the second of our two questions: was the information actually transmitted in the DNA of germ cells, or did it go by some less direct route? The fact that fewer of the second generation mice were tolerant than the first argues that the characteristic was not fixed in the germ line, and thus would require some special proviso for it to be accepted as inheritance in the DNA. In place of this, some other routes bear consideration. Could tolerance have been transmitted via a regulatory antibody or lymphocyte, or an antigen-bearing stem cell, in the seminal plasma? There is no precedent for such a route. In addition it would require the mothers to become tolerant, which seems highly unlikely in view of the known difficulty of inducing tolerance to MHC antigens in the adult animal.

Another possibility, by which one might escape frontal challenge to Weissmann's dogma, is that the information could be carried across the generations by viruses as such, without requiring violation of germ-line DNA. There are indeed a number of instances in which virus infection both *in vitro*, and *in vivo* has been associated with the appearance of unexpected (or 'alien') MHC specificities. But whether the virus actually transduces the MHC-specific information (as would be necessary if this were to explain the inheritance of tolerance) or whether it merely triggers some predetermined change in the

structure of the MHC molecules is not known. In any case there is as yet no good evidence from this or any other source for viral transduction *in vivo*. Furthermore, for viral infection to serve as an explanation it would have to be strictly limited to the venereal route, otherwise tolerance would have been expected to appear in some control mice in the same animal house.

Yet another possibility (A. Müllbacher, personal communication) depends on the already-mentioned capacity of F_1 hybrids to make a response to the parent. Thus if the tolerogenic inoculum of F_1 lymphoid cells were making such a response to the CBA host, it might have the effect of selecting against the CBA-type among developing germ cells, and thus favour any mutations bearing A strain antigens. These

mutations could then be perpetuated by normal inheritance, and there would be no need for a special viral transduction mechanism. Once again, however, there is no precedent for this type of selection.

In answer to our second question, then, it is not easy to offer an alternative mechanism for the transmission of tolerance which is any more credible than the Lamarckian one favoured by Gerczynski and Steele. Clearly, the basic result must be repeated in other laboratories. It will be important to establish mendelian inheritance through further generations, and to characterize the tolerant state. In addition one would expect from the point of view of survival value in evolution, that immune responsiveness as well as non-responsiveness would prove to be heritable. □

Solar luminosity variations

from Ronald L. Gilliland

THE solar luminosity is the primary force driving the earth's atmospheric circulation and it is probable that variations in the solar constant of as little as 0.3% would have a measurable effect on the climate. In a recent paper, Dearborn and Blake (*Astrophys. J.* **237**, 616; 1980) describe the effects on the solar luminosity of varying the efficiency of convection and they briefly discuss mechanisms which could produce such changes. One such mechanism is a fluctuation in the average properties of the convection cells seen near the solar surface. At the depth where the luminosity is quite sensitive to the convective efficiency the number of cells is $\sim 10^3$. If the properties of individual cells are allowed to vary by 10%, then stochastic variations of $\sim 0.3\%$ in the total flux could occur. An alternative mechanism for inducing changes of convective efficiency is interaction with the cyclic variation of magnetic field strength associated with the 11 year solar sunspot cycle.

To examine the significance of the Dearborn and Blake study it is necessary to briefly describe the solar convection zone. The sun has a convective region extending to a depth of some 200,000 km or $\sim 30\%$ by radius. As over most of this region convection is very efficient and capable of carrying a much larger flux than is actually present, changes in the convective efficiency will have little effect on the luminosity. In the outer 3,000 km the convective efficiency begins to drop. Here a significant part of the flux is carried by radiative transfer and changes in the convective flux are readily induced by small changes in the convective efficiency. Dearborn and Blake have adopted the

standard approach of modeling the very complex phenomena of turbulent convection with a simple 'mixing length' theory in which the size of convective cells at any given level is given by the assumed mixing length. It is the assumed mixing length which Dearborn and Blake alter to bring about a change of convective efficiency. They find that quite small changes in the mixing length parameter on timescales of days to $\sim 10^3$ years can lead to significant luminosity variations. These results are interesting and provocative since they show that solar luminosity variations are theoretically plausible on these timescales. However, quantitative predictions cannot be made as there is no physical connection between the proposed mechanisms leading to convective efficiency variations and modeling of them.

The solar models which show luminosity changes in response to a forced variation of convective efficiency also show radius variation. Sofia *et al.* (*Science* **204**, 1306; 1979) argue that measurements of solar radius variations can be used as a proxy measure of luminosity changes. The use of radius variations as a proxy depends on the theoretical determination of the relative change of radius with respect to luminosity (defined as $W = \frac{\Delta R/R}{\Delta S/S}$, R = solar radius, S =

solar constant) resulting from assumed changes in convective efficiency. This would be very useful since historical records of the solar radius (from transit timings of the sun crossing the meridian, eclipse observations, and timings of Mercury transits) extend back a few hundred years. Unfortunately, direct monitoring of the solar luminosity has been carried out only for parts of this century and there is insufficient data for long-term correlations with climate.

The early claim by Sofia *et al.* that radius

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Devonian trilobites

from R. A. Fortey

IN a recent publication*, Eldredge and Branisa describe a whole series of beautifully preserved trilobites from Devonian rocks in Bolivia, including a number of genera and species new to science.

The trilobites are an extinct group of arthropods that flourished from the Cambrian to Permian periods (about 600–250 Myr BP). Unlike many invertebrate fossils, they have a complex and varied morphology, and rapid changes have occurred in virtually all parts of the exo-skeleton. They are therefore an ideal group to use as tests of the different theories for reconstructing phylogeny. The visual system in particular is very sophisticated and there are complex shufflings in the arrangement and numbers of lenses. The specimens described by Eldredge and Branisa are remarkable for their well-preserved muscle impressions and they have been able to record speciation within a geographically and taxonomically limited group. Of particular interest is their demonstration of the number of specific variations that can be played on a relatively few morphological themes — a product of one of the rapid 'radiations' that happened from time to time during the long history of the trilobites. □



Andinacaste legrandi. Sora Sora, Bolivia. Dorsal view of cast of a complete individual $\times 3$.



Bainella (Bainella) 'acacia'. Pebble Island, Falkland Islands. Dorsal view of cephalon $\times 1$.



Phacopina. Belén region, Bolivia. Dorsal view of portion of thorax $\times 3$.

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*Niles Eldredge & Leonardo Branisa *Calmonioid Trilobites of the Lower Devonian Scaphioecia zone of Bolivia, with remarks on related species* Bulletin of the American Museum of Natural History 165, 2; 1980.

variations could be used as a means of determining luminosity changes has encountered several problems. First of all, similar calculations conducted by different groups have found widely discrepant values for W . (Dearborn and Blake found $W = 5 \times 10^{-3}$. Sofia *et al.* found $W = 7.5 \times 10^{-2}$. In my own studies I have found $W = 8.5 \times 10^{-4}$. This is a total spread of $\times 100$!) The numerical discrepancies are related to a much more fundamental problem. The mixing length theory does not model the turbulent convective flow in a time-dependent deterministic sense (feedback with variations of individual cells or the magnetic field can not be included). The mixing length is a free parameter which is normally adjusted in order for the calculation to match time-averaged global observed conditions (for example, radius and luminosity). While the mixing length theories may adequately describe time-independent global structure, results of perturbations within the mixing length theory framework do not necessarily have any relation to actual physical processes. The mixing length theory does not adequately describe the detailed structure of the outer 3,000 km of the solar convection zone (where the changes in question arise). Indeed different versions of mixing length theory exist

which give observationally indistinguishable solar models, but quite different structures in the outer 3,000 km which are so important for this problem. Using mixing length theory to determine more than rough qualitative estimates for the relative radius and luminosity variations is highly questionable.

In order to predict reliable quantitative results for solar radius and luminosity variations it will be necessary to directly incorporate the causal mechanisms. For the stochastic variation mechanism a deterministic hydrodynamic model of convection in multiple dimensions is required. To follow the effects of a variable magnetic field one must couple the full 3-dimensional hydrodynamic treatment of convection with an equation to determine the magnetic field. One must thus solve the non-linear astrophysical dynamo problem (see Moffat, H.K. *Nature* 285, 16; 1980). This is a very difficult problem — reliable quantitative results are unlikely to be found in the near future.

While past measurements of solar luminosity and radius variations have not been highly significant, our ability to make precise measurements may be expected to improve in the near future. The Solar Maximum Mission launched February 14, 1980 should provide measurements of the

solar luminosity with an absolute accuracy of 0.1% and a relative precision 0.02% over a planned period of 1–2 years. Preliminary results indicate that the quoted precision will be achieved and that statistically significant variations are occurring. Willson *et al.* (*Science* 207, 179; 1980) have claimed direct detection of a 0.4% luminosity increase over a 2.4 year period with two rocket flights. A change of this magnitude occurring periodically over a few years or as part of a longer trend would have probable climatological significance. SMM and future satellite missions should allow us to know whether or not solar luminosity variations are taking place. The High Altitude Observatory is sponsoring a solar diameter measuring project based on transit timings which should be capable of detecting monotonic radius changes to $< 0.01\%$ over a baseline of a few years. Simultaneous radius and luminosity determinations are of importance in developing and checking theoretical models of solar variations. Thus, while current theoretical models of solar variations can be viewed as little more than suggestive, the day is near when direct observational determinations of such changes can be made, which in turn should help the development of realistic models. □

Pseudogenes

from Nick Proudfoot

A new and unexpected discovery has now come from the molecular analysis of gene families that code for globin. Studies from a variety of mammalian species reveal that in each case more gene sequences are present in the genome than are necessary to encode the known globin polypeptides. Sequence analysis of these extra globin genes shows that they have a variety of nucleotide changes that would result in either aberrant or absent polypeptide formation. The term 'pseudogene' — defined as a region of DNA that displays significant homology to a functional gene but has mutations that prevent its expression — has been coined for these aberrant genes.

The first example of a eukaryotic pseudogene was described by Jacq, Miller and Brownlee (*Cell* **12**, 109; 1977) for the *Xenopus* 5S gene system. These and further studies (Miller *et al.* *Cell* **13**, 717; 1978) revealed that the 5S gene was directly adjacent to an almost but not quite faithful 5S gene copy. No pseudogene 5S RNA has ever been detected *in vivo*. More recently extra genes which may be pseudogenes have been found in the rabbit (Hardison *et al.* *Cell* **18**, 1285; 1979) and human β -globin gene clusters (Fritsch *et al.* *Cell* **19**, 959; 1980) and human α -globin gene cluster (Lauer *et al.* *Cell* **20**, 119, 1980).

During a recent meeting entitled "Haemoglobin Switching" at Airlie House, Virginia, US the β -like gene families of human, rabbit, mouse, sheep and goat and α -like gene families of human and mouse were described in some detail. In each case more genes were detected than expected and evidence was presented suggesting that most of these extra genes are, in fact, pseudogenes. As recently published from the laboratories of Phillip Leder and Oliver Smithies (Nishioka *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 2806; 1980; Vanin *et al.* *Nature* **286**, 222; 1980), Nishioka and Vanin described a mouse α -globin gene that has various frame shift mutations which are in turn followed by in-phase termination codons. As a result no α -globin-like polypeptides can be produced. The gene has a further remarkable feature — both introns are precisely excised so that it resembles a somewhat mutated α -globin messenger RNA.

Both speakers suggested possible mechanisms to explain how such an 'intronless' gene could have arisen. A favourite model is that messenger RNA or its reverse transcript somehow upsets the replicative process, even though the presence of globin mRNA or reverse

transcriptase in germline cells has yet to be demonstrated. During replication of a globin gene, messenger RNA or complementary DNA might base pair with the gene sequence and loop out the two introns. Subsequent repair of such hybrids could result in intron excision. Another, perhaps more plausible, model is that the enzymes involved in splicing out introns from precursor RNA might also be capable of splicing DNA in an analogous manner. Presumably such DNA splicing would have to be a freak occurrence, or no intron would be safe. Nishioka described a second mouse α -like globin gene that appears to be a candidate for pseudogene status. Although both introns are present, several base changes in the gene would alter amino acids thought essential for globin function. Furthermore, the normal termination codon is mutated so that translation of the mRNA would continue into the 3' noncoding region. Such a mutation has been found to be the cause of a rare genetic disorder in man — haemoglobin Constant Spring (Clegg *et al.* *Nature* **234**, 337; 1971).

Two pseudogenes of the goat were described by Lingrel in a β -globin gene family (Zingrel, Haynes, Schon, Clearly & Gallagher, unpublished). One gene has several terminator codons close to the start of the gene that would prevent the expression of globin. The other has a variety of base changes that would alter amino acids essential to globin function. There are also β -like globin pseudogenes in positions between embryonic and adult globin genes in mouse and rabbit. Hutchinson described the mouse β -pseudogene which appears to have completely lost its 5' side so that the first β -like sequence detectable is at around amino acid position 75. Beyond this the sequence is clearly β -like and the second intron is in place. However, short deletions and additions of sequence together with terminator sequences indicate that this gene is inactive (Jahn, Hutchinson, Phillips, Weaver, Haigwood, Voliva & Edgell *Cell* in the press). In contrast to the mouse gene the rabbit β -pseudogene as described by Hardison has β -like sequence corresponding to all regions of the adult β gene except for its 3' noncoding sequence which is barely identifiable. Again a deletion of one nucleotide close to the beginning of the gene coding sequence quickly puts any translation product from this gene out of phase (Lucy & Maniatis *Cell* in the press). Finally, a human α -globin pseudogene was described (Proudfoot & Maniatis *Cell* in the press), again between the embryonic and adult α -like genes. This gene is 70-80% homologous to the adult α -globin gene throughout its sequence but has a deletion of 20 nucleotides in the middle of the gene that puts the amino acid

sequence out of phase.

An interesting feature of the last four pseudogenes is that they lack the putative intron splicing signals found at the beginning and end of all introns (Breathnach *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 4853; 1978; Lerner *et al.* *Nature* **283**, 220; 1980). It therefore seems unlikely that any transcripts produced by these genes could be spliced correctly. Two further features of the globin pseudogenes described should be mentioned. First, most of them, in addition to mutations that would directly affect translation into globin polypeptides, have potential transcription mutations. For instance, the intronless mouse α -globin and the second goat β -globin pseudogenes lack the putative promoter signal or 'Hogness Box' PyATAp (M. Goldberg, Doctoral Thesis, Stanford University), while the human α -globin and the same goat β -globin pseudogenes lack the putative polyadenylation signal AATAAA (Proudfoot & Brownlee *Nature* **263**, 211; 1976). Second, in each case where the complete linkage arrangement of the particular globin gene cluster has been established (human α and β , rabbit β , mouse β) a pseudogene is found between the embryonic or fetal and adult globin genes.

Can any function be ascribed to pseudogenes? Vanin *et al.* postulate that pseudogenes might restrict gene expression by using up the transcriptional apparatus of the cell in futile pseudogene transcription. Perhaps the presence of a pseudogene next to each adult globin gene is related to such a regulatory process. Alternatively, globin pseudogenes could be the source of new gene products if translated in one of the two other coding frames or if translation includes intron sequences which are unspliced. However, so far no new protein product has been suggested or identified for these pseudogenes.

It may be better to consider pseudogenes as relics of evolution. Globin genes probably evolved by duplication. After the initial duplication event each member of a pair could diverge and useful sequence variations would be selected. Presumably, first α and β and then embryonic and fetal globin genes were produced in this manner. Mutations that restrict expression of the genes could also occur. In an extreme case, such as a promoter mutation, it is possible that one gene might be completely switched off. There would be then no selective advantage in the switched-off gene remaining globin-like so the whole gene sequence might be expected to gradually drift from the original sequence. In another case a mutation might only partially inactivate the gene resulting in low level expression. Human δ -globin and mouse β -minor globin genes might be living examples of such an evolutionary process. Finally, it is possible that certain gene mutations such as a frame shift might not

only incapacitate the gene but result in the expression of a new protein detrimental to the whole animal. Although such mutant genes might be expected to rapidly disappear from the population, further inactivating mutations might occur that could then allow these genes to become fixed in the population. There is, in any case, no reason to believe that pseudogenes *per se* have any function although, of course, new genes could evolve from them.

The molecular analysis of globin genes is probably more advanced than any other mammalian gene system at this time. It seems likely that when other gene families are equally well characterized new pseudogenes will be identified. Indeed, extrapolating from the situation in globin, one in four gene sequences may be pseudogenes. Put in simple terms this suggests that one quarter of our genes might be dead. □

Volcanic ash southern style

from Ian Smalley

WITH the eruptions of Mt. St Helens still making the headlines in the northern hemisphere this was a good time to have a conference* on volcanic ash deposits (alias tephra) in New Zealand. It might be noted that the St Helens eruption has produced about 1 km³ of ash but the last eruption of the Taupo volcano in the mid North Island of New Zealand produced about 24 km³, possibly all in about 15 minutes through a vent 100 m wide.

P. Froggatt (Victoria University, Wellington) reviewed the tephra from Taupo. There have been 8 major rhyolitic eruptions during the past 10,000 years. Their stratigraphy and distribution is well understood and provides useful marker beds over most of the North Island. A source vent for each eruption has now been established. The most useful and widespread marker bed is the Taupo pumice (1,820 yrs BP) which erupted from within Lake Taupo at Horomatangi Reefs. The stratigraphy and nature of the Taupo pumice have now been revised and show a variety of eruptive mechanisms. The last phase of the eruptions was the most voluminous and destructive, being a series of pyroclastic flows which swept radially outward over all topography for more than 60 km. Reconstruction of the eruptive history of this ignimbrite has important implications for older eruptions such as the Kawakawa tephra (20,000 yrs BP) and the older welded ignimbrites.

P. Kamp (Waikato University) discussed the Pleistocene tephra in Hawkes Bay and their implications for Quaternary events. The tephra provide an absolute time-scale for the Kidnappers Group sedimentary succession, the deposition of which was predominantly glacio-eustatically controlled and which matches the standard deep-sea chronostratigraphy (core V28–238). The correlation of Pleistocene tephra elsewhere in New Zealand with

Kidnappers tephra may enable events on land to be tied into the sea-level chronology. Nine discrete rhyolitic tephra, which Kamp has named informally A to I, occur in the 350 m thick Kidnappers Group succession.

V. Neall (Massey University) offered some comparisons between the volcanoes in the Cascade Range in the Western United States (including Mt. St. Helens) and Mt Egmont in New Zealand. The geological setting of each volcano and its relationship to non-volcanic rocks provides useful comparisons, particularly with respect to control of pyroclastic flow and lahar mudflow distribution. Frequency of tephra eruptions in Holocene times varies from 1 per 100–200 years at St. Helens (based on the last 4,000 years record), to 1 per 250–350 years at Egmont, 1 per 500–1,000 years at Rainier and 1 per 800 years at Shasta. The Egmont tephra succession displays a remarkably uniform mineralogy in marked contrast to tephra within the last 500 years. Pyroclastic flows from Egmont have been as frequent as those produced from St Helens and Shasta, but are much less in volume.

A. Palmer (Victoria University, Wellington) considered the occurrence of airfall tephra in the loess of the Wairarapa, at the southern end of the North Island. The 20,000 yr BP Kawakawa tephra occurs as a pinkish white 5–10 cm band of coarse silt to fine sand-sized glass and pumice fragments and is consistently found at two thirds the depth of the top (Ohakea) loess unit. The tephra influences bulk density and water contents for at least 50 cm above the primary airfall material and similar bulk density and water content variations within the older loesses probably indicate volcanic ash. Some further observations on loess were provided by N. Kennedy (DSIR Soil Bureau, Rotorua) but he was concerned with what has come to be known as 'tephric' loess. Some problems are

arising in the more northerly parts of the North Island with a redeposited tephra material which might be classed as a loess. According to Kennedy tephric loess is deposited during arid climatic periods and is always found sandwiched between tephra of 15,000–20,000 yrs BP. Thus the period 15–20,000 BP is regarded as cold and arid. In the Rotorua basin the average rate of accumulation during that critical period was 0.3 cm/yr.

D. Seward (N.Z. Inst. Nuclear Sci.) reported on progress in fission track dating of tephra deposits. Tephra represent time planes within both marine and continental sediments and are most suitable for stratigraphic chronology and correlation. Glass was the first material used for fission track dating of tephra but most glasses have undergone varying amounts of annealing and this has reduced the size and density of the spontaneous fission tracks, and produced a 'young' age. The most suitable mineral in the tephra is zircon. The Tertiary-Quaternary sequences in New Zealand contain sufficient tephra for a tight time scale to be realised throughout. However, because of error limits the fission track ages of individual tephra must be closely analysed before tephra from one sequence can be absolutely correlated with those from another. C. Vucetich (Victoria University, Wellington) offered some Quaternary tephra correlations, from c.40,000 yrs BP to c.300,000 yrs BP. The chronology was based on two fission track dates for zircons by B. Kohn: Puketirau tephra (Tirau) 0.22 ± 0.03 Myr and Omokoroa tephra (Tauranga) 0.36 ± 0.04 Myr and indirect dating per c.120,000 yr marine transgression.

D. Lowe (Waikato University) demonstrated the usefulness of tephra layers preserved in peats. The recent identification of more than twelve unweathered and unmixed thin discrete air-fall tephra in piston cores from shallow peaty lakes near Hamilton have provided a stratigraphic record of late Pleistocene (post-Kawakawa tephra) and Holocene tephra deposits in the Waikato region. Identification of the tephra was greatly aided by using X-radiography techniques and these are being actively developed by the Waikato group, with particular possibilities seen in the techniques of xeroradiography which utilises a reusable photo receptor plate instead of conventional X-ray film.

The situation may appear hazardous but like the farmers persistently cultivating the slopes of Etna the scientists find rich results are derived from volcanic ash. The scale of New Zealand vulcanism in the past has been colossal with the Taupo eruptions judged by G.P.L. Walker to have been perhaps among the largest ever. The enormous spread of volcanic material in New Zealand means that the datable volcanic events in the north can offer useful stratigraphic indicators even down into the glaciated south. □

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*Tephra Workshop meeting, held at Victoria University, Wellington, on 30 June and 1 July 1980. Organised by C. Vucetich, R. Howarth and P. Froggatt; proceedings to be published by the Geology Department, Victoria University.

Strategies of drug resistance in herpes simplex

from H. J. Field and G. K. Darby

ALTHOUGH effective anti-viral drugs are only just being developed it already seems likely that viruses have diverse strategies of drug resistance. Foremost among the anti-viral drugs is acyclovir (ACV)*, a nucleoside analogue which has shown great promise particularly against herpes simplex virus (HSV) infection. A wealth of encouraging results already exist for animal model systems¹⁻⁸, and clinical trials in man are now underway in Britain and the US.

Workers at Burroughs Wellcome first showed that two virus specified enzymes are involved in the mechanism of action of this compound⁹⁻¹¹. Firstly ACV is 'activated' in infected cells by conversion to its triphosphate, the initial step in this process being carried out by the virus-coded enzyme, thymidine kinase (TK). For the drug to block the multiplication of the virus, the ACV-triphosphate must then interact with a second virus-coded enzyme, DNA polymerase^{9,12}.

Elion and her colleagues⁹ showed that a virus unable to induce thymidine kinase was resistant to the drug. It also seemed that a second class of resistant virus — one with an altered DNA polymerase function — was theoretically possible. Investigations of resistant viruses generated by passage of HSV in the presence of the drug¹³ suggested that resistance could indeed arise in this way, although initially it was only observed in TK⁻ viruses. Evidence that mutations in the DNA polymerase gene of HSV could confer resistance to ACV was provided independently by Coen and Schaffer¹⁴ and Schnipper and Crumpacker¹⁵. In both cases viruses were investigated in which exposure to phosphonoacetic acid (PAA) — a compound acting predominantly at the level of DNA polymerase¹⁶ — had produced PAA resistance. The TK⁺ viruses selected for resistance to PAA had also frequently acquired resistance to ACV, and it was concluded that the most likely location for this second ACV-resistance locus was the polymerase gene. Both groups also observed that selection of resistant strains in the presence of ACV generally yielded TK-deficient viruses although Coen and Schaffer¹⁴ described one strain which appeared in addition, to have acquired resistance at the polymerase locus.

Is ACV resistance likely to become a clinical problem in man? To answer this we must look at the biological properties of resistant viruses. TK⁻ viruses arise readily in tissue culture in actively dividing cells where TK is a non-essential virus function. Evidence is accumulating, however, that such viruses are attenuated in animal model systems, particularly in their interactions

with the nervous systems^{13, 17, 21}. This may make their establishment *in vivo* unlikely and may also explain the lack of reports of such isolates from animals undergoing treatment with ACV.

In contrast with the frequent isolation of TK-deficient viruses, the change to resistance at the DNA polymerase locus occurs much less readily¹³⁻¹⁵. Since in this case the enzyme function is essential for virus replication, there must be considerable constraints on structural alterations. Nevertheless, studies on PAA-resistant viruses²² and a TK⁺, ACV-resistant virus¹³ suggest that these viruses may not be attenuated as much as TK-

deficient strains and may therefore be more of a problem. It is interesting in this context that a study by Hirano *et al.* last year²³ revealed that resistant strains of HSV isolated from patients treated with Idoxuridine were TK⁺, leading the authors to speculate that these were DNA polymerase mutants. This contrasts with the TK⁻ phenotype commonly associated with Idoxuridine resistance generated *in vitro*.

In the next few months information should become available on isolates obtained from humans treated with ACV. The *in vitro* work to date suggests that the analysis of such isolates will be complex and may well reveal unexpected strategies employed by HSV to outwit the nucleoside chemist. □

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Primeval galaxies and the X-ray background

from Beatrice M. Tinsley

YOUNG galaxies must have been spectacular objects, with very high rates of star formation and associated bright stars, supernovae, optical nebulosities and X-ray sources. Some years ago, Partridge and Peebles noted that primeval galaxies might be detectable not only as individual objects (*Astrophys. J.* **147**, 868; 1967) but also as a source of diffuse background radiation at wavelengths from ultraviolet to infrared. (*Astrophys. J.* **148**; 1968). The search for primeval galaxies began with those papers and continuing into the present has branched into X-ray astronomy.

Most of the effort has focussed on indi-

vidual primeval galaxies, with optical searches for them and theoretical models for their properties in all parts of the electromagnetic spectrum, (for a review see Sunyaev *et al. Comments Astrophys.* **7**, 183; 1978; Meier & Sunyaev *Scientific American Nov.* 1979 and Tinsley *Phil. Trans. R. Soc.* **296**, 303; 1980). Galaxies have been found in deep counts that have much bluer colors and probably higher rates of star formation than their nearby descendents (Bruzual & Kron *Astrophys. J.* in the press), but it is not known whether these are young or luminous enough to qualify as *bona fide* primeval galaxies.

Searching for the integrated background light of young galaxies has proved to be an extremely difficult task at optical wavelengths because the diffuse extragalactic

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light is fainter than the zodiacal light and probably fainter than scattered starlight in our own galaxy. Thus the latest observational efforts (Spinrad & Stone, *Astrophys. J.* **226**, 609; 1978; Dube *et al.* *Astrophys. J.* **232**, 333, 1979) have succeeded only in setting readily understandable upper limits to the integrated light of primeval galaxies.

A new idea is that young galaxies might be detectable in the X-ray background — a well-established isotropic radiation that may or may not be simply a superposition of quasars and other known X-ray sources (see Rees in *Objects of High Redshift* IAU Symposium **92**, ed. Abell & Peebles, 1980). Some astronomers have been tempted to account for the X-ray background by thermal bremsstrahlung from a hot intergalactic medium, perhaps dense enough to close the universe, but this is unsatisfactory because the energy required to heat the gas would be excessively high (Field & Perrenod *Astrophys. J.* **215**, 717; 1977). However, thermal bremsstrahlung is a possibility if the emitting gas is very highly clumped, and this is where young galaxies enter the picture: two papers by Tanaka and Ikeuchi (*Prog. Theor. Phys.* **62**, 393; 1979) and Bookbinder *et al.* (*Astrophys. J.* **237**, 647; 1980) consider the radiation from gas heated by supernovae in primeval galaxies. The supernovae would heat the gas to a temperature of about 100 keV, implying that its thermal X-ray emission could explain the spectrum of the background (at energies of about 2–50 keV) if most of the active phase of star formation in galaxies occurred rather recently in cosmic time, at redshifts less than 2.

The difficult question is whether young galaxies can supply enough X-rays to account for a significant fraction of the background intensity. Bookbinder *et al.* conclude that they can, but the point is debatable. Their ingenious approach is to estimate the X-ray emission of each young galaxy by relating its supernova production to the mass of metals produced by exploding stars; the mass of metals can then be estimated in turn from the quantity of metal-rich stars now remaining in each galaxy. The present luminosities and colours of galaxies are the observable quantities used to indicate the mass of metal-rich stars, and an uncertain factor in the calculation is the ratio of this mass to total galactic luminosity. Bookbinder *et al.* find that the emitted X-rays are sufficient if the mass-to-luminosity ratio is 90 solar units. This number is uncomfortably high, since, as the authors note, observed mass-to-luminosity ratios of the metal-rich parts of galaxies are typically about 10. Nevertheless, Bookbinder *et al.* feel that, within the uncertainties, hot has been driven from young galaxies could contribute significantly to the background near 50 keV.

This would be a very satisfying result, both accounting for the X-ray background and implying that primeval galaxies have been found. It should be noted, however, that

the predicted X-ray intensity depends on the square of the mass-to-luminosity ratio of metal-rich stars, so with the plausible value of 10 for that ratio the young galaxies contribute only 1% of the X-ray background. A similar result was obtained by Tanaka and Ikeuchi (*loc. cit.*), who scaled their result to the mean density of galactic stars in the universe, and found with a plausible density that young galaxies contributed about 1% of the X-ray background at 10 keV. A significantly higher contribution would require more supernovae in young galaxies than estimated by either set of authors, which does not seem very likely since Bookbinder *et al.*'s supernovae even accounted for the known metals in intergalactic space. It therefore seems most plausible that gas heated in young galaxies makes a negligible contribution to the X-ray background.

Primeval galaxies may also be bright in X-rays from other sources. For example,

Bookbinder *et al.* consider the possibility of large populations of stellar X-ray sources, analogous to the famous Cygnus X-1 (black hole) system in our own galaxy. However, stellar and other possible diffuse sources in primeval galaxies probably do not contribute a significant part of the background, since they could not account for its spectrum over a significant energy range.

We seem to be back to the question of whether primeval galaxies can ever be found. Individual sources may be a more promising avenue of discovery than any integrated background radiation, because they can always be studied further for predicted signatures of young galaxies. As noted above, some faint blue galaxies have been found that are viable candidates for very young objects, and it would be most exciting to study these in all possible detail, including high resolution imagery with the coming Space Telescope. □

Noisy chaos

from N. MacDonald

IN experiments in hydrodynamics the onset of turbulence (see *Nature* **283**, 431; 1980) is frequently preceded by a sequence of bifurcations from one periodic mode to another of doubled period. This has caused renewed interest in those mathematical models characterised by a similar series of solutions with period doubling, followed by the onset of non-periodic 'chaotic' behaviour as some parameter of the model is varied.

Perhaps the best known deterministic physical model with a chaotic solution is that of Lorenz (*Nature* **271**, 305; 1978) for flows in a horizontal layer of liquid across which a temperature gradient is maintained. In Lorenz' own calculations chaos comes about, as the Rayleigh number R is raised, by a sudden transition from a simple periodic flow. However, Robbins recently has shown (*S.I.A.M. J. App. Math.* **36**, 457; 1979) that as one lowers R to approach the chaotic region from the other direction, there is a sequence of bifurcations of this kind.

In a much simpler category of models employing one-dimensional maps (May *Nature* **261**, 459; 1976) such as

$$X_{n+1} = \lambda X_n (1 - X_n) \quad (1)$$

the multiple bifurcations are through solutions of period 2, 4, . . . 2^N , . . . as one increases λ . The transition values λ_N accumulate in a regular manner towards a limiting value λ_c at which a non-periodic solution first occurs. Models of this type, are commonly used to study the population dynamics of insects which have one distinct

generation each year, and one such model has recently been applied to populations of annual plants (Watkinson *J. theor. Biol.* **83**, 345; 1980). Difference equations such as (1) also have a somewhat subtle connection with differential equation models such as that of Lorenz.

There are difficulties in interpreting experimental data by means of any purely deterministic model with non-periodic, and apparently randomly fluctuating, solutions. This is perhaps most readily seen when a model such as (1) is used to describe population dynamics. The reproductive rate λ is liable to change from year to year because of random environmental influences. How can one expect to unscramble these effects from those of deterministic chaos for a fixed λ ? Again, in any physical model with such delicately poised dynamics it would be wise to allow for the perturbing effects of thermal fluctuations by including an additional random force term.

Rather little attention seems to have been paid to this difficulty, but in a recent paper Crutchfield and Hubermann (*Phys. Lett. A* **77**, 407, 1980) have studied the equation

$$X_{n+1} = X_n (1 - X_n) + N\sigma, \quad (2)$$

in which $N\sigma$ is a Gaussian random number with zero mean and standard deviation σ . They have also studied the effect of a random force term added to a model of an anharmonic oscillator with a periodic forcing term. Several authors (*Nature* **274**, 847; 1978) have studied chaos in deterministic models of this type, and Crutchfield and Hubermann have previously shown (*Phys. Rev. Lett.* **43**, 1743; 1979) that multiple bifurcations occur in their particular version. They have characterised the chaotic solutions in

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terms of a frequency spectrum containing a number of strong spikes on top of a broad distribution of frequencies. As one moves across the chaotic region, away from the critical parameter value that ends the period-doubling sequence, these spikes merge in pairs, simplifying the spectrum. Eventually only the broad band remains, the solution giving way to a simple periodic solution. The authors mention that Lorenz, in unpublished work, has established a similar structure of the solutions of model (1).

The strikingly simple qualitative result now obtained by Crutchfield and Hubermann is that, both in model (2) and in the noisily forced oscillator, the effect of the noise term is to remove the later stages of the bifurcation sequence of chaotic solutions. As the amplitude of the noise term is increased, this bifurcation gap widens.

Cystic fibrosis

from Leighton Dann

UNTIL a few years ago, the genetic disease cystic fibrosis (CF) was considered to be a childhood disease as most patients succumbed at an early age. With the use of modern drugs the prognosis has been much improved and patients commonly reach adulthood. Thus CF has graduated from the paediatric clinic and entered general medicine.

This changing pattern provided the focus for much of the 8th International Congress on the disease held recently in Toronto*, with sessions on progression with age, and on genetics, diagnosis and screening.

The basic defect, as yet unidentified, primarily affects exocrine glands and so various bodily functions are impaired. Bronchopulmonary disease remains the greatest problem, and successful treatment and control of infection is a major goal of research. Several delegates discussed alternatives to antibiotic therapy, and H.V. Reynolds (Yale University New Haven) reported the use of immunoprophylaxis. Much of the work has concentrated on the bacterium *Pseudomonas aeruginosa* as it is so prevalent and so highly pathogenic in CF lung infection, particularly in the later stages of the disease. Although he showed that immunization with a multivalent lipopolysaccharide vaccine boosted existing titres of antibodies against the organism in the blood, no effect was noted in the lungs of patients. As with antibiotic therapies, serum values of drugs or antibodies bear little relation to local concentrations in the lungs. Reynolds felt that although this

The authors believe that this result should be capable of experimental test in situations in which the noise level can be controlled. Their anharmonic oscillator model was originally devised for application to solid state systems where anharmonic degrees of freedom can couple to a periodic field. Superionic conductors are an example of such a system. They are (Boyce & Hubermann *Phys. Rep.* 51, 189; 1979) solids which display ionic conductivities many orders of magnitude greater than those of normal salts. They can be characterised by the presence of a rigid lattice of one type of ion in which another type of ion can move freely. The diffusion coefficients are very dependent on temperature, and for this reason Crutchfield and Hubermann suggest that superionic conductors may be particularly suitable for controlled variation of the noise term to test for the bifurcation gap. □

method of treatment was of no value to the patient who is already infected, early immunization, before infection with *P. aeruginosa*, might prove beneficial. He also suggested that other antigens should be studied, for example exotoxin A and several high molecular-weight cell wall polysaccharides.

A most promising therapeutic application of the chelating agent EDTA was proposed by R.E. Wood (Case Western Reserve University, Cleveland). He and his colleagues found that incorporation of EDTA into the culture medium retarded the growth of *P. aeruginosa* due to sequestration of divalent metal ions, particularly magnesium. There was also marked synergism shown between EDTA and many commonly used antibiotics. Preliminary experiments on animals show that EDTA does not affect mucociliary transport at the concentrations needed to arrest growth of bacteria and may therefore prove safe for use in the human lung.

Another major problem of CF is pancreatic insufficiency. Although it may be controlled by administration of pancreatic extracts, large and often increasing amounts must be given as the disease progresses. A new form of enzyme supplement, Pancrease, (manufactured by Johnson & Johnson, Slough, UK) was discussed by R. Dinwiddie (Hospital for Sick Children, London) A. M. Weber (Hospital Ste-Justine, Montreal) and D. J. Holsclaw and H. Keith (Hahnemann Hospital, Philadelphia).

Pancrease consists of enteric coated

microspheres of pancreatic enzymes in a gelatin capsule, and passes intact through the stomach to the intestine. With this mode of administration fat absorption is greatly improved and patients have access to a more normal diet as well as being able to take fewer tablets each day (about 6 instead of about 30). Similarly, better food absorption was reported by D.J. Cameron (Hospital for Sick Children, London) and E. Rossi (Childrens Hospital, University of Berne) if cimetidine is used as an adjunct to pancreatic supplements. This drug lowers gastric acid output and so higher concentrations of enzymes reach the intestine intact.

Early diagnosis is of paramount importance for good prognosis, and much effort was focussed on newer methods of detection, especially those potentially amenable to mass screening or to antenatal diagnosis. R.B. Ellio (University of Auckland) described his test for immunoreactive trypsin in newborns. Because the pancreatic ducts are blocked, trypsin leaks into the blood and is present in high concentrations in affected babies. Great interest was shown in this test, as it is done on dried blood spots and could therefore be incorporated into existing screening programmes for phenylketonuria.

J. Lieberman (Veterans Administrations Medical Centre, Sepulveda, California) described an assay which detects a specific IgM-binding fructose-specific lectin in CF serum. He suggested that this test could prove useful in detecting heterozygote carriers of the CF gene, as the lectin is present in the blood of parents of affected children. The connection between this lectin and the CF factor (a highly basic small protein found in many body fluids) remains to be elucidated.

J.C. Manson (Western General Hospital, Edinburgh) reported the production of antiserum in mice using CF factor isolated from serum by isoelectric focusing. Preliminary results with the antiserum are highly successful but its availability is limited. This method has potential as a screening test for homozygotes not only in newborns but also antenatally, for amniotic fluid from an affected fetus contains CF factor. In another approach to antenatal diagnosis, reported by L.G. Dann (Queen Charlotte's Hospital, London), an arginine esterase assay is used on cultured amniotic fluid cells. However lack of suitable samples is hampering validation of the method.

While delegates were optimistic that the outlook for patients with CF would continue to improve, it was clear that a better understanding of the disease and in particular, the basic defect, was needed before CF became truly a treatable disease. The work in progress presented at the congress encourage the hope that by the time of the next congress, in Britain in 1984, much of this will have been achieved. □

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*The Conference was held on 26th-30th May 1980 in the Royal York Hotel, Toronto and was organized by the Canadian Cystic Fibrosis Foundation.

ARTICLES

Free calcium in heart muscle at rest and during contraction measured with Ca^{2+} -sensitive microelectrodes

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Direct measurements of free Ca^{2+} in heart cells are needed for an understanding of the regulation of contractility. We developed and used Ca^{2+} -sensitive microelectrodes with fine tips, stable properties and ample sensitivity to free Ca^{2+} in the sub-micromolar range. In quiescent ventricular muscle, measurements which passed tests for electrode sealing and cell viability gave a mean free Ca^{2+} concentration of $0.26 \mu\text{M}$. During contractures, we recorded Ca^{2+} transients rising as high as $10 \mu\text{M}$. In studying the effects of catecholamines on free Ca^{2+} and force, we found evidence that adrenaline can reduce myofibrillar Ca^{2+} sensitivity in intact heart muscle.

ALTHOUGH it is widely believed that a rise in intracellular calcium activates contraction in heart muscle, the level of free Ca^{2+} at rest or during activity remains quantitatively uncertain. The strength of contraction can be widely varied by ions, hormones or drugs. It is often not clear, however, whether such inotropic agents act by changing the amount of activator Ca^{2+} or by modifying the Ca^{2+} -responsiveness of the contractile machinery. Direct measurements of free Ca^{2+} in cardiac muscle are obviously desirable. Recently, the Ca^{2+} -sensitive photo-protein aequorin has been used to record Ca^{2+} -related signals from various cardiac preparations¹⁻³. The technique is well suited for monitoring changes in Ca^{2+} during twitch contractions, and has already provided much useful information about inotropic effects. However, as the investigators are careful to point out, the method has certain limitations. The light signals do not easily give absolute values of free Ca^{2+} because of various uncertainties, including interference from intracellular Mg^{2+} or H^+ . A large number of cells must be injected with aequorin, resting levels can apparently not be detected, and light signals during twitches must be signal-averaged to overcome noise. Ca^{2+} -sensitive microelectrodes⁴⁻⁶ offer another approach with different advantages and limitations, which may complement optical techniques using aequorin or other Ca^{2+} -indicators. In the first attempts at applying Ca^{2+} -electrodes to cardiac tissue, investigators^{7,8} used sheep Purkinje fibres because the cells in this preparation are large enough ($40\text{--}50 \mu\text{m}$ diameter) to tolerate electrode tip diameters of $\geq 1 \mu\text{m}$. To be useful in the smaller cells of working heart muscle itself ($10\text{--}15 \mu\text{m}$ diameter), Ca^{2+} -electrodes must have tips of sub-micrometre size, while retaining stable properties and ample resolution down to 10^{-7}M free Ca^{2+} or lower. These requirements are fulfilled by the Ca^{2+} -sensitive microelectrodes which we have made with a new Ca^{2+} -sensor. The electrodes enabled us to measure intracellular free Ca^{2+} in ventricular muscle, both at rest and during contractures.

Unbranched papillary muscles, $0.4\text{--}0.8 \text{ mm}$ in diameter and $2\text{--}4 \text{ mm}$ long, were isolated from right ventricles of $8\text{--}12$ -week-old ferrets (*Mustela putorius furo*). Ferrets were chosen because their hearts usually contain two or more robust preparations with suitable geometry for force measurements⁹. Muscles were placed in a chamber for superfusion with oxygenated Tyrode solution containing (in mM): 150 NaCl , 4 KCl , 1.8 CaCl_2 , 0.5 MgCl_2 , 5 glucose and $10 \text{ Tris HCl buffer}$ ($\text{pH } 7.2\text{--}7.4$). The

preparation was mounted with one end fixed and the other end attached to a force transducer. To reduce movements which would tend to dislodge the electrodes, the muscle was carefully positioned on a pedestal, with resting length well below that giving peak developed twitch force. Suitable high input impedance electrometers (WP Instruments) recorded signals from the Ca^{2+} -sensitive microelectrode (V_{CaE}) and a conventional microelectrode filled with 3 M KCl (V_{m}). Bath potential was defined as zero for both electrodes; the bath was earthed by an agar bridge containing 3 M KCl . The preparation was first penetrated by the conventional microelectrode, usually during continuous stimulation of twitch contractions. After a stable V_{m} impalement had been obtained, stimulation was discontinued and the preparation was impaled by the Ca^{2+} -electrode at a second site several cell lengths away from the V_{m} impalement. V_{CaE} , V_{m} and their difference ($V_{\text{CaE}} - V_{\text{m}} = V_{\text{diff}}$) were recorded along with contractile force on a chart recorder and a magnetic tape recorder. V_{diff} is a measure of free Ca^{2+} inside the cell.

Electrode construction and characteristics

Micropipettes were pulled from thin-walled, borosilicate tubing to a size that gave resistances of $8\text{--}12 \text{ M}\Omega$ if filled with 3 M KCl . The pipettes were baked dry at 200°C , siliconized with tri-butylchlorosilane vapour, and back-filled under pressure with the $p\text{Ca } 7$ buffer solution described in Fig. 1 legend. The electrodes were then bevelled until the resistance dropped to about 40% of the original value, giving a sharp tip with an outer diameter of about $0.5 \mu\text{m}$. Next, a $10\text{--}50\text{-}\mu\text{m}$ column of the Ca^{2+} -sensor, dissolved in tetrahydrofuran, was sucked into the tip of the electrode. This sensor, a modification of the polyvinylchloride (PVC) macroelectrode membrane of Ammann *et al.*¹⁰, consisted of 10% (w/w) neutral Ca^{2+} -ligand, N,N' -di(11-ethoxycarbonyl)undecyl- N,N' ,4,5-tetramethyl-3,6-dioxaoctane-1,8-diamide, 8% tetraphenylarsonium tetrakis(p -biphenyl)borate, 10% PVC and 72% (o -nitrophenyl)octyl-ether. The tetrahydrofuran, used to reduce viscosity, evaporated away in a few minutes, leaving a PVC gel impregnated with the other sensor components in the tip of the electrode.

This new PVC-gelled sensor offers various advantages over previous Ca^{2+} -sensitive microelectrode compositions. (1) Mechanical stability is much improved over liquid sensors; the

Table 1 Free Ca^{2+} concentration in unstimulated muscles

Preparation	Ca ²⁺ electrode no.	Penetration no.	Test(s)	pCa
Y22-2	26	3	18Ca, 30K	6.3
Y24-3	8	2	18Ca	6.7
		4	18Ca	6.4
Y25-3L	17	1	18Ca	6.6
		2	18Ca, 16K	6.4
	19a	1	18Ca	6.5
		4	18Ca, 16K	7.0
Y26-1	0	1	18Ca	6.7
	13	1	18Ca	6.9
Y29-1	7	1	18Ca, 30K	6.6
		6	18Ca, 30K	6.5
Y30-1	19b	2	30K	6.5
Mean $\pm$ s.e.m. 6.59 $\pm$ 0.06				

Intracellular pCa values were determined by comparing V_{diff} levels, taken while muscles were bathed in 1.8 mM Ca^{2+} , 4 mM K^{+} Tyrode solution, with electrode calibrations, carried out immediately after withdrawal from the muscle. Impalements satisfied quantitative criteria described in the text for electrode stability, sealing-in and cell viability. During tests of common-mode rejection using 16 or 30 mM K_o , ($\Delta V_{\text{diff}}/\Delta V_m$) ranged between 0.98 and 1.07.

both 21 and 37 °C, using Ca^{2+} buffer compositions adjusted for temperature according to data from ref. 12. Raising the temperature merely scaled the response of the electromotive force (e.m.f.) by the ratio of absolute temperatures, in accord with the Nernst equation. For values of e.m.f. obtained in resting cells, the temperature correction amounts to about 3 mV, opposite and approximately equal to the magnitude of the sodium correction. The response time of the electrodes is many seconds at micromolar levels of Ca^{2+} , so they are not expected to follow transients during single twitches. Attention was therefore focused on 'resting' levels and slow changes during maintained contractures.

Intracellular measurements in resting muscles

Figure 2 illustrates electrode recordings during penetration and withdrawal, along with some tests of the validity of intracellular measurements. Figure 2a shows V_{CaE} during impalement of a cell with a Ca^{2+} -sensitive microelectrode. V_{CaE} undergoes a rapid negative deflection due in part to the membrane potential, and a slower negative-going change which can be attributed to sealing-in of the electrode and its sluggish response at low free Ca^{2+} . Figure 2b shows V_{CaE} during withdrawal of the Ca^{2+} electrode after a continuous 3-h impalement. The main point is the comparison between steady e.m.f.s measured at the beginning and end of the intracellular recording. The steady levels measured in the tissue agree extremely well, as do the levels in the external solution, indicating the absence of significant d.c. drift.

If ($V_{\text{CaE}} - V_m$) is to be taken as a measure of intracellular free Ca^{2+} , it is necessary to consider the seal around the tip of the Ca^{2+} electrode and the viability of the impaled cell. Two types of test were applied to screen out defective impalements. One test uses changes in external potassium concentration (K_o) to vary the membrane potential over the range below contractile threshold. If the Ca^{2+} -electrode records from a cell which retains its normal resting potential, it should respond to a moderate elevation of K_o with a voltage change (ΔV_{CaE}) which closely matches the depolarization measured elsewhere in the preparation by the conventional microelectrode (ΔV_m). This is the case in Fig. 2c during a depolarization produced by 30 mM K_o . The quality of the common-mode rejection is also evident on the V_{diff} trace, which remains quite steady. Measurement of contractile force (T) confirmed that this level of depolarization was insufficient to activate tension.

In another test of impalement quality, the extracellular calcium concentration (Ca_o) was elevated, as shown in Fig. 2d. If the seal around the electrode tip was poor, or if the cell was otherwise damaged, one would expect a large V_{CaE} signal, as was sometimes seen. If, on the other hand, the tip was well sealed within a viable cell, one would expect only a modest change in

V_{CaE} . In the experiment illustrated in Fig. 2d, a 10-fold increase of Ca_o produced little change in V_m and only a small positive-going deflection in V_{CaE} . The maximal change in V_{diff} was 4 mV, corresponding to $\Delta p\text{Ca} = 0.22$, but there was no measurable development of force.

In these experiments and others, measurements of free Ca^{2+} in unstimulated muscles were examined according to various criteria to decide whether or not the impalements were acceptable. (1) Electrode stability was assessed in each case by comparing pre-impalement and post-impalement calibrations. The measurement was rejected unless the responses to pCa 6 agreed to within 10 mV. (2) The quality of the seal around the Ca^{2+} -electrode tip and the viability of the impaled cell were checked by one or both of the following tests. First, exposure to 18 mM Ca_o (as in Fig. 2c): measurements were rejected if V_{diff} changed by more than 10 mV. Second, exposure to 16 or 30 mM

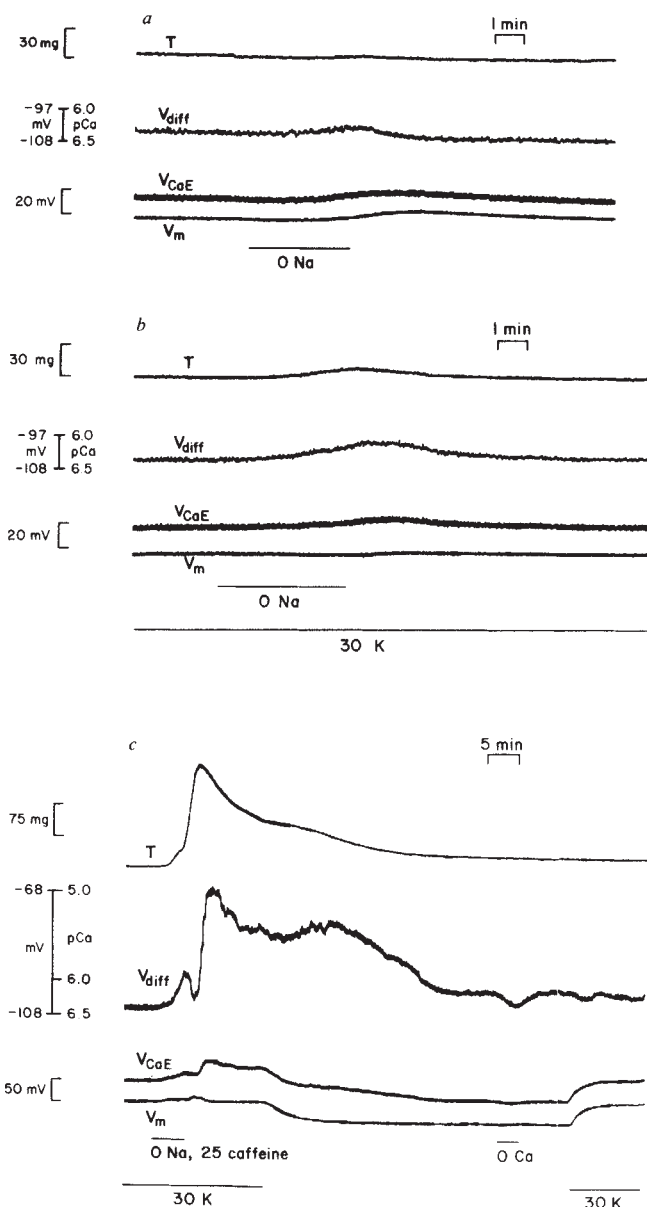


Fig. 3 Changes in free Ca^{2+} and force in response to a series of zero Na^{+} challenges during continuous V_{CaE} and V_m impalements. *a*, Exposure to 0 Na(choline) in the presence of 4 mM K_o . V_m was -92 mV at beginning of record. *b*, 0 Na(choline) exposure in the presence of 30 mM K_o . V_m was -48 mV at beginning of record. *c*, Exposure to 0 Na(choline), 25 mM caffeine in the presence of 30 mM K_o . K_o was reduced from 30 to 4 mM and later restored to 30 mM as a test of whether both electrodes would respond equally to an imposed membrane potential change. Exposure to nominally Ca^{2+} -free solution was used to check continued responsiveness of V_{CaE} .

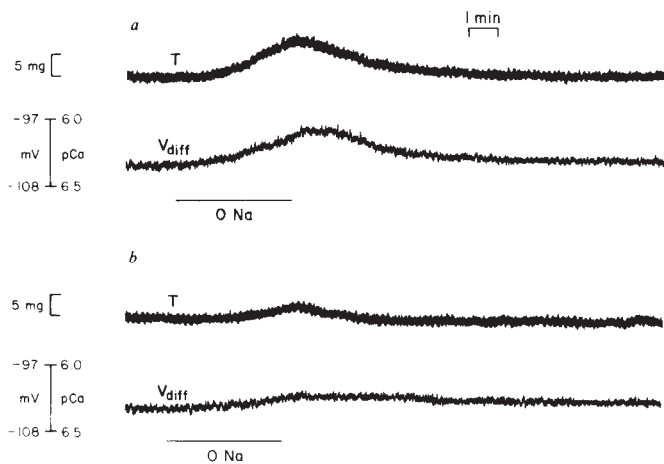


Fig. 4 Effect of isoprenaline ($3 \mu\text{M}$) on Ca^{2+} and force signals produced by Na^+ withdrawal in the presence of 30 mM K_o . Relative to control (a), Ca^{2+} and force transients were both attenuated by the catecholamine (b) and showed partial recovery towards their original amplitudes when a third 0 Na (choline) challenge was applied 25 min after removing the drug (records not shown). Records in a are taken from the same run as those in Fig. 3b.

K_o (see Fig. 2d): measurements were rejected if ΔV_{CaE} and ΔV_m differed by more than 10%. From a large number of attempts, 12 measurements were considered acceptable by these criteria (Table 1). The average resting level in $p\text{Ca}$ units was 6.59 ± 0.06 (mean $\pm$ s.e.m.), or $0.26 \mu\text{M}$. In the same series of measurements, the average resting potential in 4 mM K_o was $-84.7 \pm 1.7 \text{ mV}$.

Calcium transients during contractures

How does the free Ca^{2+} in unstimulated muscles compare with the level needed to activate the contractile proteins? One indication comes from the above-mentioned test of raising extracellular Ca^{2+} from 1.8 to 18 mM . Although any resulting large rises in intracellular Ca^{2+} were discarded due to the likelihood that gross penetration damage had occurred, small increases (as in Fig. 2c) were nevertheless observed even in five runs where independent tests with 16 or 30 mM K_o indicated good electrode sealing (Table 1). In those runs, raising extracellular Ca^{2+} 10-fold gave a peak increase of 1.2–2.6-fold in intracellular Ca^{2+} , but no measurable force ever developed. This suggests that resting levels were safely below the threshold for Ca^{2+} -activated force development in the conditions of our experiments. Such a conclusion may not hold at longer sarcomere lengths, corresponding to the peak of the length-tension relationship^{20–22}, where the myofibrils are known to be more sensitive to Ca^{2+} (refs 23, 24).

The relationship between free Ca^{2+} and force was studied further during sodium-removal contractures. Figure 3 shows records from a continuous Ca^{2+} -electrode impalement illustrating the responses to progressively stronger contracture challenges. Figure 3a shows the effect of replacing extracellular Na^+ with choline in the presence of 4 mM K_o . Here, Na^+ removal elicited an apparent 40% rise in free Ca^{2+} , but only a barely detectable increase in force. (Because the presumed fall in intracellular Na^+ would have depressed V_{diff} by 1 to 2 millivolts, the true rise in Ca^{2+} would have been slightly more than 40%). In Fig. 3b the stimulus for increasing intracellular Ca^{2+} was strengthened by partially depolarizing the preparation with 30 mM K_o before Na^+ removal, a procedure known to enhance Na^+ -free contractures. In this case, the intracellular Ca^{2+} rose to a peak of almost $1 \mu\text{M}$. ($1 \mu\text{M Ca}^{2+}$ is high enough for changes in intracellular Na^+ to have a negligible effect on the electrodes.) Finally, in Fig. 3c, the contracture challenge was further augmented by including 25 mM caffeine in the Na^+ -free solution. This produced a much larger force transient, and a Ca^{2+} signal which reached a peak of $10 \mu\text{M}$. To demonstrate that the V_{CaE}

signal was not due to dislodgment of the Ca^{2+} electrode, K_o was changed. Both V_{CaE} and V_m display similar hyperpolarizations during reduction of K_o from 30 to 4 mM , as well as the corresponding subsequent depolarization when 30 mM K_o was restored. The quality of the common-mode rejection, seen at higher amplification on V_{diff} , argues that the Ca^{2+} -electrode remained well sealed within a viable cell. The three parts of the experiment demonstrate the feasibility of recording both small and large calcium transients during varying degrees of contractile activation.

Relaxant effects of catecholamines

The advantages of the Ca^{2+} -electrode approach encouraged us to study the regulatory effects of catecholamines on excitation-contraction coupling. Stimulation of β -adrenoreceptors by adrenaline or isoprenaline not only increases developed force during a twitch (the positive inotropic effect) but also abbreviates the twitch and accelerates relaxation. These 'relaxant effects' (refs 25–29) are important in allowing adequate time for diastolic filling when the heart rate increases, as with exercise. A closely related relaxant phenomenon can be seen during contractures when exposure to adrenaline or isoprenaline reduces the force evoked by K^+ -induced depolarization^{25–27} or Na^+ withdrawal²⁶. Two possible mechanisms involving cyclic AMP^{28–30} have been suggested by studies of subcellular fractions or of skinned or hyperpermeable preparations. First, cyclic AMP may increase Ca^{2+} uptake by the sarcoplasmic reticulum^{31,32} and, second, it may decrease the Ca^{2+} sensitivity of the myofibrils by promoting phosphorylation of a regulatory protein such as troponin-I^{33–36}. Contracture experiments with Ca^{2+} -electrodes offer the possibility of seeing whether either or both

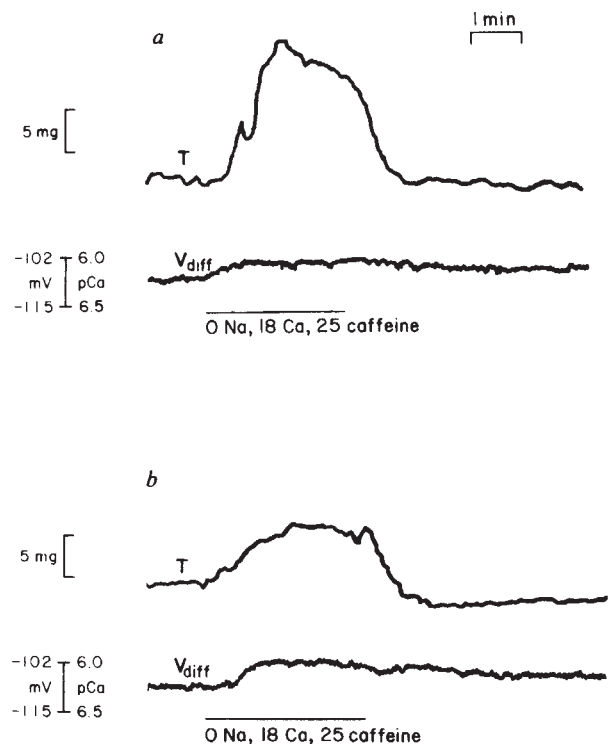


Fig. 5 Effect of adrenaline on Ca^{2+} and force signals evoked by caffeine-containing contracture solution. Horizontal bars indicate period of superfusion with 0 Na (choline), 18 mM Ca , 25 mM caffeine . 30 mM K_o present throughout. a, Control; b, $10 \mu\text{M}$ adrenaline (7 min). Contracture force returned to within 25% of control when the challenge was repeated after 20 min superfusion with adrenaline-free solution containing $1 \mu\text{M}$ propranolol but the Ca^{2+} -electrode was unfortunately dislodged by the contracture. The force records are unusually noisy in this figure because a force transducer with low sensitivity and low compliance was used in the hope of minimizing preparation movement.

mechanisms operate in intact cardiac muscle. Figure 4 shows Ca^{2+} and force signals during Na^{+} -withdrawal contractures: Fig. 4a is the response to Na^{+} -free solution before drug treatment, and Fig. 4b is the response in the presence of 3 μM isoprenaline. Both the force transient and the Ca^{2+} transient were reduced by the catecholamine (and showed partial recovery when the drug was removed). Similar results were obtained in an experiment using 10 μM isoprenaline. Because the Ca^{2+} transients, as well as force, were attenuated, these observations are consistent with enhancement of Ca^{2+} uptake by the sarcoplasmic reticulum as the predominant relaxing mechanism, although other possibilities are not excluded.

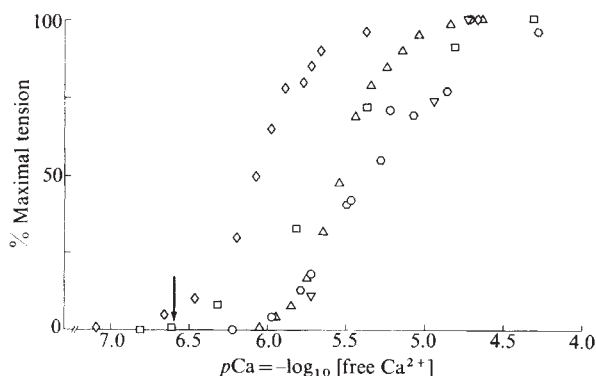


Fig. 6 Calcium requirements for cardiac myofibrillar activation, taken from published work on skinned or hyperpermeable preparations of mammalian cardiac muscle. All data have been adjusted along the horizontal axis so that recalculated $p\text{Ca}$ values conform with Ca-EGTA binding constants given in ref. 12. The same binding constants were used in preparing calibration buffers for the Ca^{2+} -electrodes¹¹ and give log (effective stability constant for Ca-EGTA at pH 7.0) = 6.42. This value is in close agreement with the value (6.40) determined by Allen *et al.*⁴². $\diamond$, Solaro *et al.*⁴³, Fig. 3, triangles. $\circ$, Fabiato and Fabiato⁴⁴, Fig. 7, inverted triangles. $\circ$, Kerrick and Donaldson⁴⁵, Fig. 1, filled triangles. $\square$, Endo and Kitazawa⁴⁶, Fig. 7. ∇ , McClellan and Winegrad³⁵, Fig. 9, open circles. Δ , Hibberd and Jewell²⁴, Fig. 1, filled circles. The arrow denotes the mean $p\text{Ca}$ (6.59 ± 0.06) in quiescent myocardium as determined in this study.

Any reduction of myofibrillar Ca^{2+} sensitivity could best be seen if the Ca^{2+} transient were not reduced. To this end, caffeine was included in the contracture solution to inhibit Ca^{2+} uptake by the sarcoplasmic reticulum and promote net release³⁷. As Fig. 5a, b illustrates, adrenaline treatment actually increased the Ca^{2+} transient during a 0 Na^{+} /18 Ca^{2+} /25 caffeine challenge. However, the contracture force was reduced, as expected if the contractile machinery were less responsive to a given level of Ca^{2+} . This result was confirmed in each of two additional experiments using 10 μM adrenaline. These observations provide evidence that adrenergic regulation of myofibrillar Ca^{2+} sensitivity, first proposed on the basis of biochemical studies, can indeed occur in intact cardiac muscle.

Discussion

Our experiments show that Ca^{2+} -sensitive microelectrodes can be made sharp enough and selective enough to measure free Ca^{2+} inside relatively small mammalian cells. The bevelled tips of these electrodes are sufficiently fine for impalements to be maintained with no greater difficulty than those of ordinary voltage-recording microelectrodes, despite the strong contractility of cardiac ventricular muscle. The nearly logarithmic response of the Ca^{2+} -electrode to free Ca^{2+} makes it possible to quantify intracellular Ca^{2+} levels both at rest and during contraction, with only slight interference from other ions. Limitations of this technique include the rather slow speed of response at the low intracellular Ca^{2+} levels, and the requirement for simultaneous measurement of membrane potential. Also, a Ca^{2+} -electrode takes a point sample which could be

unrepresentative of Ca^{2+} concentrations elsewhere. Gradients inside one small ventricular cell are unlikely to persist over the time scale examined in the present work, but we have seen marked variations in the pattern of Ca^{2+} responses in different cells in the same papillary muscle. Thus, it is not surprising that the tension recorded from the whole muscle often fails to track the Ca^{2+} transient, which was always measured in a single superficial cell. When studying modifications of contractile responses, we have assumed that physiological or pharmacological interventions have qualitatively similar effects on all the cells.

Our mean resting level of free Ca^{2+} , 0.26 μM ($p\text{Ca}$ 6.59) in ferret ventricular muscle, is somewhat lower than that reported by Coray *et al.*³⁸, 0.36 μM in sheep myocardium, but higher than results of Uhm *et al.*³⁹, 0.12 μM in rabbit ventricular muscle. Over the critical range 0.1–1.0 μM free Ca^{2+} , the liquid sensor electrodes of those workers had a smaller response (5–9 mV)^{8,38} than the PVC-gelled electrodes of the present study (18–24 mV), so that any small errors in estimates of the membrane potential or of interference from other ions would have less influence on our values for $p\text{Ca}$. Although the present measurements are not limited by electrode sensitivity to Ca^{2+} , all microelectrode recordings are susceptible to artefacts from impalement damage. We have accepted only those measurements which passed above-mentioned criteria for good electrode sealing and cell viability, but some residual perturbation by penetration damage cannot be excluded.

It is interesting to compare Ca^{2+} levels measured in intact fibres with levels which produce tension in 'skinned' or 'hyperpermeable' mammalian ventricular preparations, where Ca-EGTA buffers are applied directly to the contractile apparatus. Figure 6 shows $p\text{Ca}$ -tension relationships from six published reports, adjusted for a standardized Ca-EGTA dissociation constant. Our resting value of $p\text{Ca}$ 6.59 (vertical arrow) is at, or in most cases well below, the apparent threshold for Ca^{2+} -activated force. Moreover, the peak Ca^{2+} readings in the series of challenges shown in Fig. 3 are $p\text{Ca}$ 6.3, 6.1 and 5.0. Reading off from the main cluster of $p\text{Ca}$ -tension relationships for skinned fibres, we would expect barely detectable, small and near-maximal tensions, respectively, and indeed, the observed peak tensions were ~0, 9 and 240 mg. This agreement should be viewed with caution because of the microelectrode sampling problem discussed above and because skinned fibre $p\text{Ca}$ -tension relationships can be markedly influenced by temperature^{40,41}, solution composition³⁵, sarcomere length^{23,24} and perhaps, method of skinning and source of tissue. The effects of adrenergic compounds on Ca^{2+} transients and contractures were also in qualitative agreement with results from subcellular fractions^{31–34,36} or hyperpermeable preparations³⁵. Working with intact muscle, we found evidence that catecholamines may modify myofibrillar Ca^{2+} sensitivity and reduce peak Ca^{2+} elicited by sodium removal. These results illustrate how Ca^{2+} electrodes could be useful in future studies on the regulation of cardiac contractility.

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Note added in proof: Lee, Uhm and Dresdner⁴⁷ have just described Ca^{2+} -electrode measurements in rabbit ventricular muscle. Their Ca^{2+} values incorporate a calculated activity coefficient of 0.32 and are based on an effective stability constant for Ca-EGTA of $10^{6.76} \text{ M}^{-1}$ at pH 7.0 rather than $10^{6.42} \text{ M}^{-1}$ as in our work. Multiplication by 6.84 corrects their data to our units of free Ca^{2+} concentration and brings their value of 38 nM Ca^{2+} activity in resting cells to 260 nM free Ca^{2+} concentration, in fortuitously exact agreement with our results. O'Doherty *et al.*⁴⁸ have recently reported another sensor composition which appears to have comparable performance to the mixture used here.

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Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome

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We have studied the organization of immunoglobulin heavy-chain genes in a $\gamma 2b$ -chain (BALB/c allotype)-producing myeloma BKC F_1 #15 induced in a F_1 mouse between C57BL and BALB/c. Southern blot hybridization studies using cloned μ , $\gamma 1$ and $\gamma 2b$ -chain genes as probes demonstrate that the μ - and $\gamma 1$ -chain genes of the expressed chromosome are deleted while these genes of the unexpressed chromosome are retained. The $\gamma 2b$ -chain gene of the expressed allele is rearranged while that gene of the unexpressed allele seems unchanged, as do the $\gamma 2a$ -chain genes. These results support the allelic deletion mechanism in heavy-chain class switch and the order of H chain genes.

DREYER and Bennett¹ proposed a model that a variable region (V) and a constant region (C) of the immunoglobulin polypeptide chain are encoded by two separate genes in the germ-line, which are eventually brought together by somatic recombination. Recent isolation and characterization of immunoglobulin light (L)- and heavy (H)-chain genes have convincingly borne out the predictions of the Dreyer-Bennett hypothesis^{2–11}. Analyses of L-chain genes have demonstrated that the V–C recombination takes place between a germ-line V_L gene and a J_L region that is a few thousand nucleotides away from the C_L gene^{12,13}. It is believed that the intervening sequence separating the J and C genes is removed at the RNA level by splicing¹⁴.

Possible mechanisms have been proposed to account for the V–C recombination, which can be classified into deletion, copy-insertion, excision–insertion and inversion models^{15–19}. A model for the immunoglobulin gene organization and reorganization should explain such unique phenomena known in lymphocytes as allelic exclusion and class switch. In a given lymphocyte a C_H gene of one allele is expressed and that of the other allele is suppressed (allelic exclusion). A V_H -region sequence is successively joined with different C_H -region sequences during differentiation of a lymphocyte (class switch).

Previously we showed that the copy numbers of the specific C_H genes in myeloma DNA were reduced depending on the class or subclass of the C_H gene expressed in the myeloma²⁰. Our data indicate that the DNA segment located between a V_H gene and the C_H gene expressed is excised out of the chromosome to recombine the two genes. Such recombination seemed to occur only on one of the two allelic chromosomes. We have also

proposed that H-chain genes are aligned on one chromosome in the order: V_H genes, unknown spacer, μ , $\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2a$ and α (ref. 20). Such alignment, together with successive deletion, can explain the class-switch phenomenon. This model is called the allelic deletion model. The deletion of C_H genes, in accord with the above order of the C_H genes, was recently confirmed by Rabbitts *et al.*²¹. The deletion of the DNA segment located between two adjoining sequences was also confirmed in the κ -type L-chain gene by Sakano *et al.*¹².

Elsewhere we have reported cloning of the rearranged $\gamma 1$ -chain gene from a $\gamma 1$ chain-producing myeloma MC 101 and compared its structure with H-chain genes cloned from newborn mouse DNA²². Such analyses indicate that the rearranged $\gamma 1$ -chain gene contains at its 5' flanking region a newly introduced segment that was located originally at the 5' flanking region of the μ -chain gene in newborn mouse DNA. The recombination site is not the putative J region but a novel region called S region. These results are in agreement with the prediction of the deletion model, although they do not necessarily prove the model.

In this report we describe direct evidence that the deletion of the specific H-chain genes takes place only on the expressed chromosome using a myeloma induced in a F_1 mouse between C57BL and BALB/c which have different allotypes in the H-chain C regions.

Probes and restriction maps of the μ -, $\gamma 1$ - and $\gamma 2b$ -chain genes

We have already cloned the *EcoRI* fragments containing the $\gamma 1$ -, $\gamma 2b$ and μ -chain genes from newborn mouse DNA and determined their nucleotide sequences^{4,8,9,22–24}. We used the 1.2-kilobase pair *HindIII* fragment of the cloned μ -chain gene,

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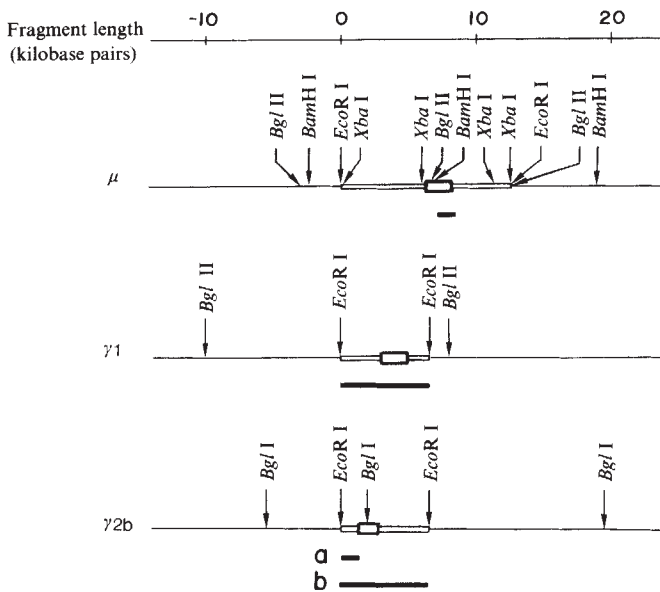


Fig. 1 Restriction enzyme cleavage sites around the μ -, $\gamma 1$ - and $\gamma 2b$ -chain genes. DNA is displayed from left to right with the direction of transcription. Narrower rectangles show the cloned DNA fragments. Wider rectangles show locations of structural genes. DNA fragments used for probes are indicated by horizontal bars below the restriction maps. μ -chain gene probe, 1.2-kilobase pair *HindIII* fragment that contains the CH3 and CH4 domains of the μ -chain gene clone²¹; $\gamma 1$ -chain probe, 6.6-kilobase pair *EcoRI* fragment containing the $\gamma 1$ -chain gene⁸; $\gamma 2b$ -chain gene probe a, 1.35-kilobase pair, *BamHI* fragment (5' fragment) of the $\gamma 2b$ -chain gene clone²²; $\gamma 2b$ -chain gene probe b, 6.6-kilobase pair *EcoRI* fragment containing the $\gamma 2b$ -chain gene²².

the cloned *EcoRI* fragment (6.6 kilobase pairs) of the $\gamma 1$ -chain gene, the cloned $\gamma 2b$ -chain gene and the 1.35-kilobase pair *BamHI* fragment of the cloned $\gamma 2b$ -chain gene as probes for hybridization (Fig. 1). Although the whole $\gamma 2b$ -chain gene clone ($\gamma 2b$ probe b) has extensive homology with the $\gamma 2a$ -chain gene^{23,25}, the 1.35-kilobase pair *BamHI* fragment ($\gamma 2b$ probe a), which corresponds to the 5' fragment of the cloned $\gamma 2b$ -chain gene, does not cross-hybridize with the $\gamma 2a$ -chain gene under the stringent washing conditions used in the present study. Detailed maps of restriction endonuclease cleavage sites within these clones have been determined^{8,22-24}. We have determined the restriction sites of *BglII* adjacent to the cloned $\gamma 1$ -chain gene fragment by digestion of mouse DNA with several combinations of two restriction enzymes, followed by Southern blot hybridization of the restriction DNA fragments. Similarly, we have determined the restriction sites of *BglI* adjacent to the cloned $\gamma 2b$ -chain gene fragment and the sites of *BamHI* and *BglII* adjacent to the cloned μ -chain gene fragment. These restriction sites were confirmed by Southern blot hybridization using the 5' or 3' fragment of the cloned DNA as a probe. Figure 1 summarizes the results of these analyses and the known sites of restriction endonucleases used in the present study. The fragments used for probes are also shown.

Allelic deletion of the μ - and $\gamma 1$ -chain genes

To prove the deletion of the C_H gene from one chromosome, it is essential to distinguish the C_H genes on different chromosomes. BALB/c and C57BL mice have different allotypes in H-chain C regions²⁶, suggesting that their C_H genes may have different nucleotide sequences. We searched for restriction endonucleases that produce the C_H -gene fragments of different lengths between BALB/c and C57BL alleles. Among restriction endonucleases tested (*PvuII*, *BamHI*, *EcoRI*, *HindIII*, *SmaI*, *PstI*, *KpnI*, *XbaI*, *BglII*) we found that *BglII* can distinguish the $\gamma 1$ -chain genes of BALB/c and C57BL alleles. As for the $\gamma 2b$ -chain gene, we have tested *XhoI*, *AvaI*, *SallI*, *XbaI*, *BglII*, *BamHI*, *BstEII* and *BglI*, and found that *XhoI* or *BglI*

digestion produces different $\gamma 2b$ -chain gene fragments between BALB/c and C57BL DNAs. The μ -chain genes of C57BL and BALB/c alleles were distinguished by digestion with *XbaI*, *EcoRI* or *KpnI*, while digestion with *BamHI* or *BglI* did not distinguish the two alleles.

Only a few myelomas are available, which were induced in the F₁ mouse between BALB/c and C57BL (ref. 27). BKC F₁ # 15, that produces the $\gamma 2b$ -chain protein with the BALB/c allotype, is suitable for our purpose since the allelic deletion model predicts the deletion of the $\gamma 1$ - and μ -chain genes from the BALB/c chromosome of this myeloma.

When BALB/c and C57BL DNAs were digested with *XbaI*, blotted and hybridized with the μ -chain probe, they produced the 4.8 and 2.8-kilobase pair bands, respectively (Fig. 2a, lanes 1

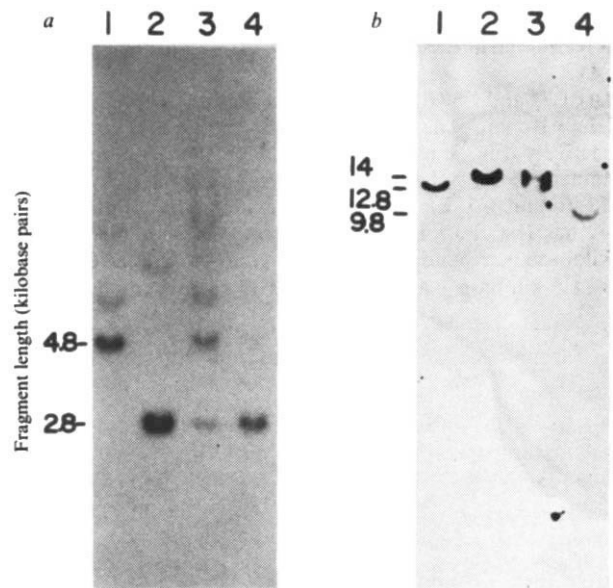


Fig. 2 Hybridization of mouse liver and F₁ myeloma DNAs with the μ -chain gene probe. Myeloma BKC F₁ # 15, (kindly supplied by Dr M. Potter) was propagated in F₁ mice between female BALB/c and male C57BL/6. BKC F₁ # 15 was induced in F₁ mice between C57BL and BALB/c and shown to produce IgG2b (ref. 27). The allotype of the BKC F₁ # 15 protein was determined to be BALB/c type by Drs K. Hayakawa and K. Okumura in Prof. T. Tada's laboratory (University of Tokyo). Mouse livers or myeloma tumours were homogenized in 50 mM Tris·HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl with a Potter-Elvehjem homogenizer at 0 °C. Sodium dodecylsulphate to 0.3% and NaClO₄ to 0.5 M were added to a whole homogenate. The homogenate was extracted with 0.5 vol. each of water-saturated phenol and a chloroform-isoamyl alcohol mixture (24:1). The water phase was separated by centrifugation, dialysed against 0.1 × SSC (0.015 M NaCl, 0.0015 M Na citrate) for 24 h digested with RNase (100 μg ml⁻¹) in a dialysis bag for 24 h at room temperature and then extracted with phenol. After dialysis against 0.1 × SSC for 48 h, 1.27 g of CsCl was added to 1 ml of the water phase. After centrifugation at 30,000 r.p.m. for 48 h, fractions with high viscosity were collected and dialysed against 0.1 × SSC for 48 h. DNAs (5 μg each) were digested with *a*, *XbaI* or *b*, *EcoRI*, electrophoresed in 0.5% agarose gels (Sigma type I) and transferred to nitrocellulose filters (Schleicher and Schuell) according to the method of Southern³². DNA fixed on filters was hybridized with the μ -chain gene probe as described previously⁸. The probe DNA was labelled with [α -³²P]TTP (Amersham) by nick translation³³. The specific activity of the probe was 200 c.p.m. pg⁻¹. After hybridization, filters were rinsed with 2 × SSC and then washed in 0.1 × SSC plus 0.1% SDS (four times) for 40 min each at 67 °C. Filters were finally rinsed with 2 × SSC, dried and exposed to X-ray film using a DuPont Cronex lightening plus intensifier screen at -80 °C. Origins of DNA used are: lanes 1, BALB/c liver; lanes 2, C57BL/6 liver; lanes 3, F₁ mouse liver; lanes 4, BKC F₁ # 15 myeloma.

and 2). BKC F₁ #15 DNA, when digested with *Xba*I, yielded only the μ -chain gene fragment of the C57BL allele while F₁ mouse DNA contained the μ -chain gene fragments of both BALB/c and C57BL alleles (Fig. 2a, lanes 3 and 4). Faint bands larger than 6 kilobase pairs are due to contamination of other *Hind*III fragments of the μ -chain gene clone to the 1.2-kilobase pair *Hind*III fragment used as a probe (see Fig. 1) because these faint bands disappeared when the recloned 1.2-kilobase pair *Hind*III fragment was used as a probe. It is worth noting that a faint band (at 6.1 kilobase pairs) of the BALB/c chromosome is also absent from the F₁ myeloma DNA. The μ -chain gene of the C57BL allele in BKC F₁ #15 appeared to be unchanged at its 3' distal region since DNAs of C57BL and BKC F₁ #15 produced identical μ -chain gene fragments of 12- and 8.5-kilobase pairs when digested with *Bam*HI and *Bgl*II, respectively (ref. 28 and see Fig. 1). However, the 5' region flanking the μ -chain gene of the C57BL allele in BKC F₁ #15 is rearranged because the μ -chain gene fragment (9.8-kilobase pairs) in *Eco*RI-digested BKC F₁ #15 DNA was smaller than the band (14-kilobase pairs) observed in *Eco*RI-digested C57BL DNA (Fig. 2b, lanes 2 and 4).

BALB/c and C57BL DNAs, when digested with *Bgl*II, yielded the γ 1-chain gene fragments of 18- and 14.5-kilobase pairs, respectively (Fig. 3, lanes 1 and 2). Naturally, F₁-mouse DNA contained the γ 1-chain gene fragments derived from both BALB/c and C57BL alleles. It is evident that BKC F₁ #15 DNA has lost the γ 1-chain gene of the BALB/c allele (the 18-kilobase pair band), whereas the gene on the C57BL allele (the 14.5-kilobase pair band) seems to remain intact (Fig. 3, lane 4).

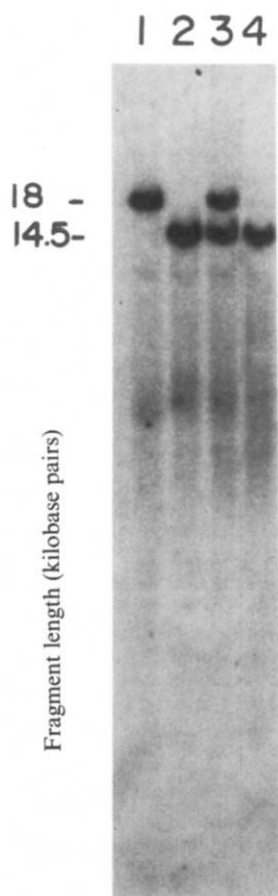


Fig. 3 Hybridization of mouse liver and F₁ myeloma DNAs with the γ 1-chain gene probe. DNAs (30 μ g each) were digested with *Bgl*II and hybridized as described in the legend to Fig. 2 with the [α -³²P]-labelled γ 1-chain gene probe. Origins of DNA used are: lane 1, BALB/c liver; lane 2, C57BL/6 liver; lane 3, F₁ mouse liver; lane 4, BKC F₁ #15 myeloma.

Table 1 Restriction fragments of immunoglobulin H-chain genes in mouse liver and myeloma

Origin of DNA	Size of restriction DNA fragments (kilobase pairs)			
	μ (<i>Xba</i> I)	γ 1 (<i>Bgl</i> II)	γ 2b (<i>Bgl</i> I)	γ 2a (<i>Eco</i> RI)
BALB/c liver	4.8	18.0	7.5	23.0
C57BL liver	2.8	14.5	9.5	23.0
F ₁ liver	4.8+2.8	18+14.5	—	—
BKC F ₁ #15	2.8	14.5	9.5 and 6.5	23.0

The results of Figs 2–4 are summarized. Restriction enzymes used are shown in parentheses.

Rearrangement of the γ 2b-chain gene of the expressed allele

The γ 2b-chain gene in BKC F₁ #15 DNA was analysed by digestion with *Bgl*I and Southern blot hybridization using the 5' fragment of the cloned γ 2b-chain gene (γ 2b probe a) as a probe. As shown in Fig. 4 (lanes 1 and 2), *Bgl*I digestion of BALB/c and C57BL DNAs produced the fragments of 7.5- and 9.5-kilobase pairs respectively, which hybridized with the γ 2b chain probe a. BKC F₁ #15 DNA, when digested with *Bgl*I, yielded the γ 2b-chain gene fragments of 6.5- and 9.5-kilobase pairs (Fig. 4a, lane 4). The results indicate that the 5' region of the γ 2b-chain gene on the BALB/c allele was rearranged, while that gene on the C57BL allele seems to remain unchanged in BKC F₁ #15 DNA. However, we do not exclude the possibility that the γ 2b-chain gene on the C57BL allele may have undergone some rearrangements which we did not detect. We also found that the 3' region flanking to the γ 2b-chain gene of the BALB/c allele is rearranged in BKC F₁ #15 DNA when the 3'-portion probe of the γ 2b-chain gene was used²⁸.

Presence of the γ 2a-chain genes

Both BALB/c and C57BL DNAs, when digested with *Eco*RI, produced the 6.6- and 23-kilobase pair fragments hybridizing to the γ 2b-gene probe b which cross-hybridizes with the γ 2a-chain gene (Fig. 4b, lanes 1 and 2). The 6.6-kilobase pair fragment corresponds to the cloned γ 2b-chain gene and the 23-kilobase pair fragment was identified as the γ 2a-chain gene fragment²¹. F₁-mouse and BKC F₁ #15 DNA contained the γ 2a-chain gene fragment (23-kilobase pairs) (Fig. 4b, lanes 3 and 4). The γ 2a-chain gene seems to be intact in BKC F₁ #15 although we cannot exclude the possibility that the γ 2a-chain gene contains an undetected rearrangement.

Organization of immunoglobulin H-chain genes in BKC F₁ #15 myeloma

Restriction DNA fragments of the μ -, γ 1-, γ 2b- and γ 2a-chain genes in BALB/c mice, C57BL mice, F₁ mice and BKC F₁ #15 myeloma are listed in Table 1. It is evident that BKC F₁ #15 has lost the μ - and γ 1-chain genes of the BALB/c chromosome and contains the rearranged γ 2b-chain gene of the BALB/c chromosome. This myeloma contains the μ -, γ 1- and γ 2b-chain genes of the C57BL chromosome. BKC F₁ #15 contains the γ 2a-chain gene although we did not distinguish two alleles. These results are in agreement with the prediction of the allelic deletion model. Nevertheless, we found an unexpected rearrangement at the 5' region flanking the μ -chain gene of the C57BL chromosome that is unexpressed in BKC F₁ #15. The two chromosomes of BKC F₁ #15 are schematically represented in Fig. 5.

Unexpected rearrangements in BKC F₁ #15

Although the deletion profile of C_H genes in BKC F₁ #15 DNA is as predicted by the allelic deletion model, we found two unexpected rearrangements in BKC F₁ #15 DNA; one in the 5'

region flanking the μ -chain gene of the unexpressed chromosome (Fig. 2b, lane 4) and the other in the 3' region flanking to the $\gamma 2b$ -chain gene of the expressed chromosome²⁸.

It is possible that rearrangement in the 5' region flanking the μ -chain gene of the C57BL allele is related to the mechanism to suppress the H chain genes on the C57BL chromosome. It may be relevant to this assumption that the 5' region flanking the μ -chain gene is proposed to contain the putative J region and the S region that is important to class-switch recombination²².

It is difficult to understand why the rearrangement in the 3' region flanking the $\gamma 2b$ -chain gene of the expressed chromosome is necessary to express the $\gamma 2b$ -chain gene. It may be possible that the 3' rearrangement of the C_H gene takes place so that the class switch stops at this particular gene. Otherwise, a V_H gene together with a J region moves farther down to a C_H gene remaining at the 3' side of the expressed gene.

Deletion of C_H genes in other myelomas

Rabbitts *et al.*²¹ reported that C_H genes, which are located 5' to the expressed C_H gene, are absent from both chromosomes in several myelomas induced in BALB/c mice. We also observed similar deletion from both chromosomes and found that the expressed C_H genes are rearranged in different manners between homologous chromosomes in these myelomas²⁸. The results may suggest that C_H gene deletion, which accompanies the rearrangement of the expressed C_H genes, takes place successively, but not simultaneously, in the allelic chromosomes. Several forms of rearranged κ -type L-chain genes were isolated from κ -chain-producing myelomas^{29,30}.

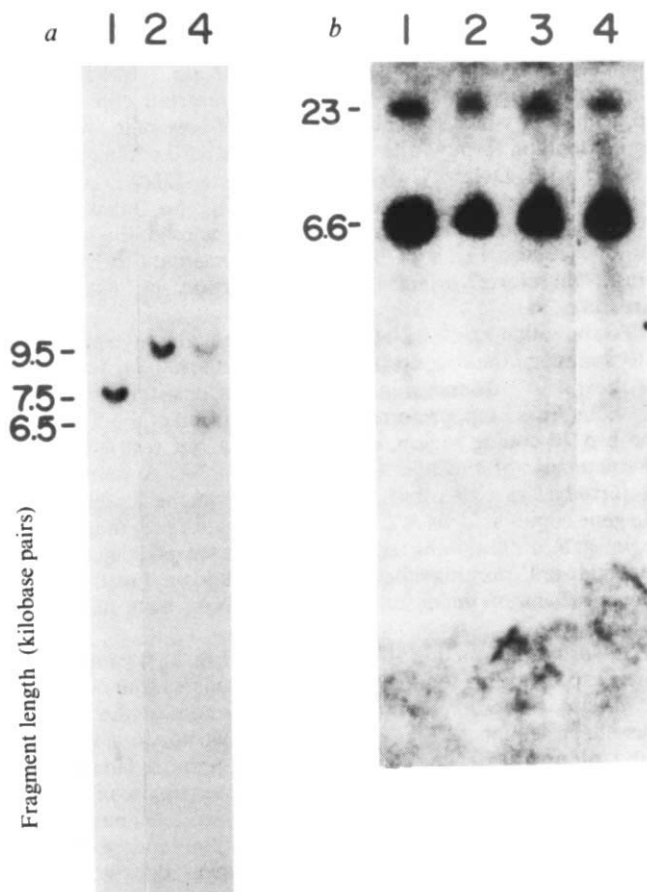


Fig. 4 Hybridization of mouse liver and F_1 myeloma DNAs with the $\gamma 2b$ -chain gene probes. DNAs were digested with *a*, *Bgl*I or *b*, *Eco*RI, and hybridized as described in the legend to Fig. 2 with [α -³²P]-labelled probes indicated. The $\gamma 2b$ probes *a* and *b* were used in *a* and *b* respectively. Origins of DNA used are: lanes 1, BALB/c liver; lanes 2, C57BL/6 liver; lanes 3, F_1 mouse liver; lanes 4, BKC F_1 #15 myeloma.

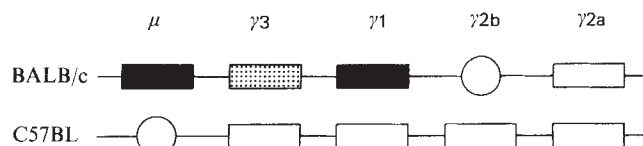


Fig. 5 Schematic representation of C_H genes in BKC F_1 #15. Each line shows one chromosome derived from each parent. The order of C_H genes is as proposed before²⁰. Open and closed rectangles indicate 'intact' and deleted genes, respectively. (The word 'intact' simply means that the change is not detected in the present study.) Circles indicate rearranged genes. The $\gamma 3$ -chain gene, which was not determined in the present study, is assumed to be deleted from the BALB/c chromosome and indicated by a dotted rectangle.

However, we cannot exclude the possibility that such rearrangements could be secondary mutations in myelomas. We are quite aware of the fact that chromosomal anomalies are rather frequent in myeloma cells²⁹. Nonetheless, the perfect agreement of the C_H -gene deletion profile (the order and allelic chromosome) with the prediction of the allelic deletion model is something more than accidental coincidence. Seidman and Leder⁵ reported that only one member of an allelic pair of L-chain genes is rearranged in κ -chain-producing myelomas, MOPC 149 and MOPC 41. Studies on differentiated B lymphocytes are necessary to determine whether or not the case of BKC F_1 #15 is universal and directly applicable to normal lymphocytes.

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The 5' ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I

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Many specific sites in Drosophila chromatin are hypersensitive to DNase I. The positions of such sites were mapped along the regions of the genome coding for two heat shock proteins. Such sites lie at the 5' ends of heat shock genes and may function as elements for recognition by molecules which regulate gene activity.

EUKARYOTIC DNA is packaged as chromatin, a complex of protein and DNA within the cell nucleus (see ref. 1 for review). How might this compaction affect the expression of genetic information? Recently, in a study of the chromatin structure at specific regions in the *Drosophila* genome, we reported that certain localized sites were exquisitely sensitive to the nucleolytic enzyme DNase I (ref. 2). By digesting native chromatin gently with DNase I, electrophoresing the DNA on agarose gels and visualizing specific regions by hybridization with a cloned probe³, we could show that each of five *Drosophila* loci displayed a unique set of preferred cleavages². This preference did not appear on naked DNA, nor in the cleavage pattern of the entire genome². Here, I map such DNase I-sensitive sites near the genes for two heat-inducible polypeptides in *Drosophila melanogaster*.

Mapping DNase I-sensitive sites

I mapped the positions of DNase I-sensitive sites by a novel indirect end-labelling technique that displays the partial digestion products. After gentle treatment of chromatin in nuclei with DNase I, the purified DNA is cleaved completely with a rarely cutting restriction enzyme, electrophoresed and blotted on to nitrocellulose in a typical Southern transfer³. The blot is then probed with a short cloned ³²P-labelled DNA segment that abuts a chosen restriction cut; the lengths of the labelled subfragments greater than the probe length define the distance between the restriction cut and the partial DNase I cuts.

This technique was applied to the genes for two heat shock proteins in *D. melanogaster* (Oregon R) embryos and in cultured cells [Schneider line 2 (ref. 4)] derived from such embryos. In *Drosophila*, an elevation in temperature from 25 °C to near 37 °C rapidly activates a small number of specific genes, and sharply reduces pre-existing gene activity (see ref. 5 for review). Cytologically, new puff sites appear in the salivary gland polytene chromosomes^{6,7}. The heat-induced messenger RNAs direct the synthesis of new polypeptides, the heat shock proteins⁸⁻¹⁴, present in all tissues tested as well as in some cell lines.

The most abundant heat-induced product is a 70,000 molecular weight heat shock protein, hsp 70. There are five copies of the uninterrupted 2.2-kilobase pair hsp 70 coding sequence, two at the cytogenetic locus 87A and three at 87C (refs 15-25). The five copies of the coding region and an adjoining 0.3-kilobase pair 5'-noncoding region are closely conserved; however, the sequences near the 5' terminus diverge somewhat between the genes at the two loci, reflected in characteristic restriction site differences^{20,24}. At 87A, the two genes lie head to head, an inverted repeat around a central spacer²⁵. At 87C, a single hsp 70 gene lies at a distance, not yet determined, from an array of two (or three, in a variant) tandem copies organized in a direct repeat¹⁹⁻²³.

Figure 1a shows the partial DNase I cuts in Schneider cell chromatin upstream from the internal *Bam* site common to all five hsp 70 gene copies; the fragmentation pattern is aligned following the left to right, 5' to 3' convention. The *Bam* digest alone, on purified DNA, is shown in Fig. 1a, lane 2. Fragments obtained in low yield in this and subsequent similar digests are due either to cross-reaction with partially homologous sequences²⁴ or to polymorphism in the organization of the sequences adjacent to the hsp 70 coding region. (Populational polymorphism in these cultured cells is not surprising. Schneider initiated the line with explants from several hundred embryos⁴; the subcultures used here have to my knowledge never been cloned.) Increasing extents of partial DNase I cleavage on chromatin (Fig. 1a, lanes 3-5) reveal preferred cuts at two adjacent sites which span about 150 and 90 base pairs, flanking and extending through the 5' end of the hsp 70 coding region. The control DNase I cleavage on naked DNA reveals a fairly uniform fragmentation pattern (Fig. 1a, lanes 7, 8). Although some sequence specificity²⁶ can be seen, this effect is minor (see also Fig. 4 of ref. 2). Hypersensitivity to DNase I must, therefore, primarily be a function of chromatin structure.

To show that no sensitive regions lie near the internal *Bam* site, I mapped the cuts upstream from an internal *Sal* site close to the hsp 70 3' end (Fig. 1b, lane 2). Consistent with the previous *Bam* map, preferred cuts are localized at the 5' end of the hsp 70 coding region, overlapping the *Sal* restriction cut characteristic of the two genes at locus 87A. A number of preferred cuts are also observed upstream from the 5' regions for the gene copies at locus 87C. Some of these sites may map to the region at 87C containing repeated sequences responsible for the heat-induced, nontranslated RNA of unknown function^{17,27}. The locations of the 5' ends of these RNAs have not been determined.

Downstream from the internal *Bam* site (Fig. 2), no sensitivity in the hsp 70 coding sequence can be detected near the common 3' *Sal* site, but other sensitive sites are found past the hsp 70 messenger sequence (Fig. 2, lane 4). As two (or in a variant, three) of the hsp 70 gene copies at 87C are organized in a direct repeat¹⁹⁻²³, the 5' sensitivity in a distal gene copy would map downstream from its preceding partner (2.0-2.1 kilobase pairs from the internal *Bam* site, expected from the tandem hsp 70 map in ref. 21). No known genes occur near the two other sensitive sites. Only three out of five *Bam* fragments are selectively lost on DNase I digestion (Fig. 2, lane 5), so two hsp 70 gene copies must lack preferred cleavages downstream from their 3' ends for at least 1 and 3 kilobase pairs, respectively. The minor DNase I cleavages which characteristically occur very close to the 5' sensitive regions (Figs 1-3) coincide with positions of micrococcal nuclease cleavage (data not shown), and might reflect sensitivity of the linker regions between specifically

placed nucleosomes¹ adjacent to the hypersensitive sites (see below).

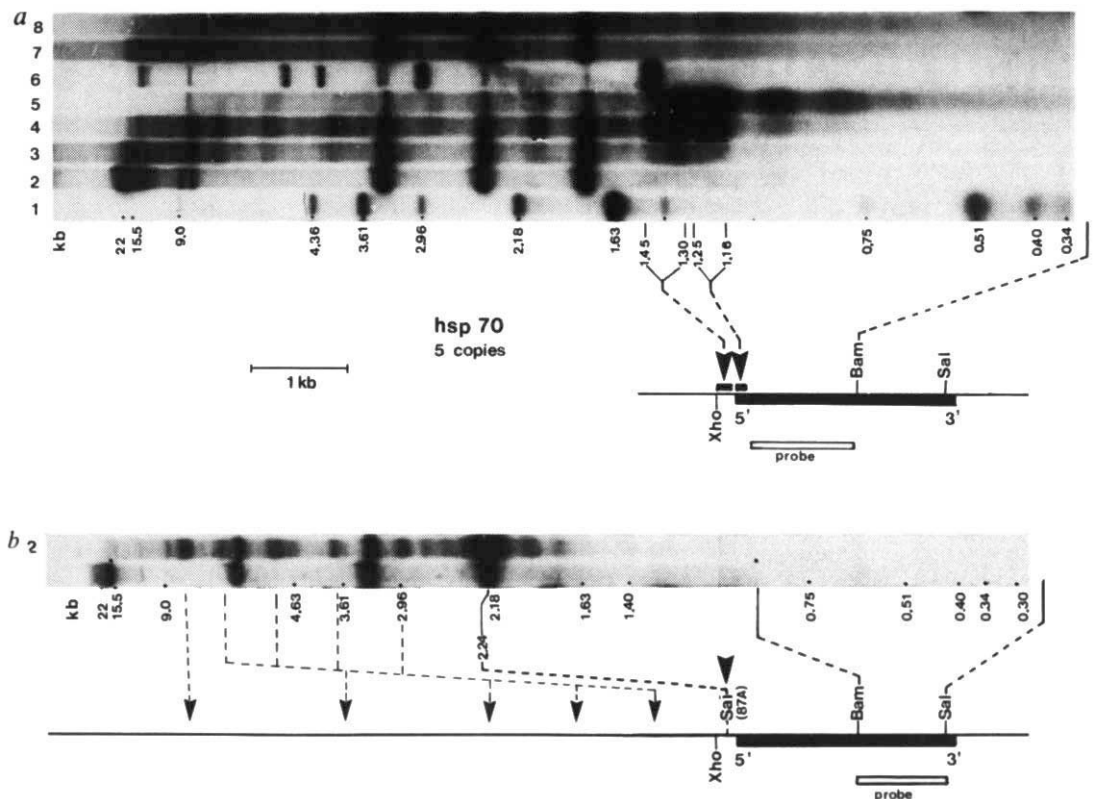
5' Sensitivity for individual hsp 70 genes

Are the 5' sensitive regions common to all gene copies of hsp 70? I exploited the restriction site differences between the genes at 87A and 87C to display selectively locus-specific DNase I cuts. By digesting with both *Sal* and *Bgl*I in the experiment shown in Fig. 3a, all fragments from 87A can be of no greater length than the 1.7-kilobase pair *Sal*-*Bgl*I segment. Thus, any longer partial fragments must be from 87C. Figure 3a, lanes 2-4, show that the 5' sensitive sites are present for the three gene copies at 87C. It is likely that this sensitivity is a sum of three individual contribu-

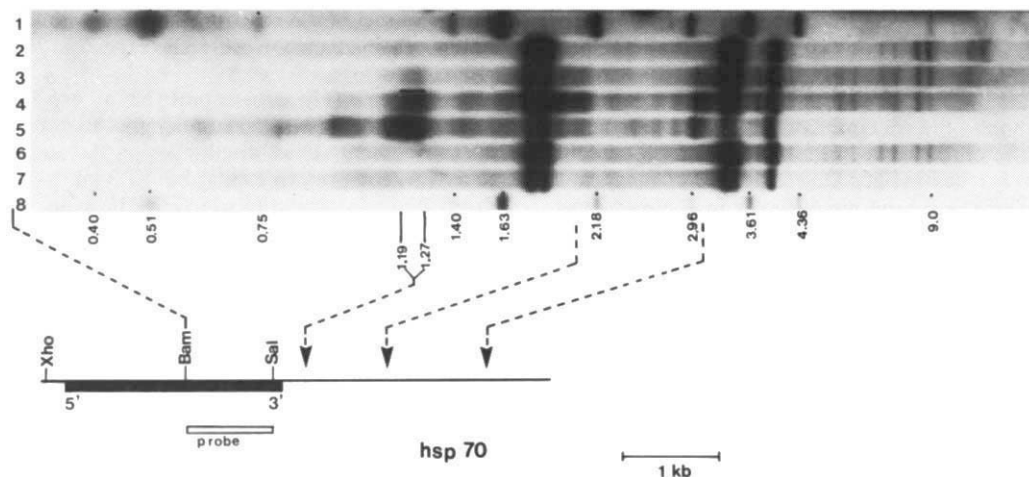
tions; otherwise, some of the 87C restriction fragments should be disproportionately retained on DNase I cleavage (Fig. 3, lane 4).

Similarly, digesting with *Pst* (Fig. 3b) ensures that all hsp 70 fragments from 87C can be of no greater length than the 1.1-kilobase pair *Pst* fragment. Thus, any longer partial fragments must be from 87A. The preferred cuts about 1.3 kilobase pairs upstream from the reference *Pst* site (Fig. 3b, lane 3) indicate 5' sensitivity for the two hsp 70 gene copies at 87A. Both must possess a 5' sensitive site, from the identical rates at which the two smallest *Bam* fragments disappear on DNase I digestion in Fig. 1a (these two fragments are specifically reduced by a *Bam* plus *Bgl*I digest not shown, to the 0.8-kilobase pair

Fig. 1 Autoradiograms showing the partial DNase I cuts in Schneider cell chromatin upstream from the internal *Bam* site (a) and the 3' *Sal* site (b) common to all hsp 70 gene copies. **a**, Nuclei were purified from *Drosophila* Schneider line 2 tissue culture cells as described elsewhere², except that the final centrifugal spin was through 1.5 M sucrose. The nuclear pellet was rinsed once before resuspension at about 3×10^8 nuclei per ml in digestion buffer² (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 0.25 M sucrose, 3 mM MgCl₂, 0.05 mM CaCl₂). Various amounts of DNase I (Worthington) were added to aliquots of nuclei and digestion proceeded for 3 min at 25 °C. The reaction was terminated by the addition of EDTA and SDS to 12.5 mM and 0.5%, respectively, and the DNA was purified by immediate organic extractions. RNA was removed by RNase A and T1 digestion, the RNases by proteinase K digestion and the protease by repeated organic extractions as described in ref. 2. The



DNA was precipitated in ethanol and redissolved at a concentration of 1 or 2 mg ml⁻¹ in 10 mM Tris-HCl pH 7.4, 5 mM NaCl and 1 mM EDTA. Secondary *Bam*HI restriction digestions were carried out according to manufacturer's directions (New England Biolabs). The digestions were terminated as above, and the DNA samples in 10% glycerol, 5% Ficoll and marker dyes were electrophoresed on a 1.3% agarose gel, 30 cm long, in a Tris-acetate buffer system⁴¹. The upper half of the gel containing the large DNA fragments was immersed in 0.1 M HCl for 15 min to facilitate transfer of the DNA out of the agarose gel⁴². The DNA was denatured, neutralized and blotted on to nitrocellulose essentially according to Southern³. The nitrocellulose filter was rinsed in 9×SSC (1×SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 0.9 M NH₄ acetate, dried and baked under vacuum for 3 h at 75 °C. The filter was presoaked in 5× Denhardt's solution⁴³ (1× Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 5×SSC, 50 mM Na phosphate, pH 7.0, and 0.25 mg ml⁻¹ denatured salmon sperm DNA for 8 h at 67 °C and hybridized for 8 h at 67 °C in a slit chamber to plasmid pW232.1 (ref. 17), labelled with ³²P by nick translation as described elsewhere² to a specific activity of about 1×10^8 d.p.m. per µg DNA. The hybridization mixture contained 5× Denhardt's solution, 5×SSC, 20 mM Na phosphate pH 7.0, 0.5 mg ml⁻¹ denatured salmon sperm DNA, 0.1% SDS and 10% dextran sulphate to accelerate the hybridization reaction⁴², and about 0.3 µg labelled plasmid DNA per 40 ml hybridization solution. After hybridization, the filter was washed twice in 5× Denhardt's solution, 3×SSC, 25 mM Na phosphate pH 7.0, 0.25% SDS and at least thrice in 0.1×SSC, 0.1% SDS, each round for 20-30 min at 67 °C. Autoradiography was on Kodak XR5 film. For later blots, the background noise could be reduced by doubling the concentration of Denhardt's solution at all stages and similarly for SDS in the primary wash. pW232.1 (ref. 17), the cloned probe segment, is homologous to the 1.1-kilobase pair (*Pst* fragment in Fig. 3 representing approximately the 5' half of the hsp 70 coding region, indicated on the restriction map by the open bar). The lanes indicate: 1, marker DNA fragments from pBR322 (ref. 44), cut with *Pst*I, *Hinf*I, *Pst*I/*Sal*I, *Pst*I/*Eco*R I, *Pst*I/*Ava*I, and other recombinant plasmid fragments homologous to pBR322 sized at 9.0, 15.5 and 22 kilobase pairs. On a gel of this agarose content (1.3%), a linear relationship between log fragment length and mobility is obtained in the region bounded by the 0.75- and 3.61-kilobase pair markers; the resolution is about 30 base pairs for fragments not overexposed. 2, *Bam* digest on Schneider cell DNA, purified directly from whole cells. An identical pattern is observed with purified DNA from isolated nuclei incubated in digestion buffer without DNase I. 3-5, Partial DNase I digests on chromatin at a final DNase I concentration of 1, 2 and 4 units ml⁻¹, respectively. Only the major regions of preferential DNase I cleavage are indicated by the arrows on the hsp 70 restriction map, aligned beneath the autoradiogram. The filled bars directly under the arrows indicate the measured span of hypersensitivity, taken from the lines drawn on the autoradiogram adjacent to lane 4. 6, *Bam* plus *Xho* digest on Schneider cell DNA. The reaction did not go to completion. 7, 8, Partial DNase I digests on naked DNA. DNA at 0.4 mg ml⁻¹ was digested with DNase I at 0.1 and 0.3 units ml⁻¹, respectively, for 1 min at 25 °C before secondary *Bam* cleavage. These two digestion points were picked from a series of purposes for comparison against the chromatin digests in lanes 4 and 5 because of their approximately equivalent overall extents of DNase I cleavage, monitored by gel electrophoresis and ethidium bromide staining after DNase I cleavage (data not shown). 9, See legend to a for a description of materials and methods. *Sal*I was used for the secondary cleavage; the DNA fragments were sized on a 1.2% agarose gel; pW229.1 (ref. 17), the cloned probe, is homologous to the *Bam*-*Sal* fragment representing approximately the 3' half of the hsp 70 coding region. The lanes indicate: 1, *Sal* digest on Schneider cell DNA. The 2.24-kilobase pair *Sal* fragment is due to the hsp 70 gene copies at 87A. 2, Partial DNase I digest on chromatin, as in a, lane 4. The hsp 70 gene copies at 87A are reduced to the 2.24-kilobase pair *Sal* fragment or shorter fragments, so all longer fragments must be due to DNase I cleavage upstream from the hsp 70 coding regions at 87C. The more prominently cleaved sites are indicated by the arrows over the restriction map at about 3.0, 3.8, 4.7, 6.1 and 8.0 kilobase pairs from the 3' *sal* site. Interestingly, the preferred cuts about 3 kilobase pairs upstream from the 3' *Sal* reference site map to a common region of homology upstream from the hsp 70 coding region; what Moran *et al.*²⁰ refer to as the x element. See text for discussion of the other sensitive sites.



DNase I digestion. From densitometry of these fragments in lanes 2 and 5, they are about half as sensitive to DNase I as the other three *Bam* fragments (data not shown). See text for discussion. 6, 7, Partial DNase I digests on naked DNA, as in Fig. 1a, lanes 7, 8.

fragment characteristic of the 87A hsp 70 sequences). In summary, the data indicate that a 5' sensitive structure is present for most or all of the hsp 70 gene copies.

The other set of preferred DNase I cuts in Fig. 3b, lane 3, is likely to be wholly a result of the inversely repeated arrangement²⁵ of the hsp 70 genes at 87A. Its interpretation is complicated by the variable length of the spacer region between these two genes²⁵, reflected in the *Pst* restriction pattern. Assuming that the 2.6–2.9-kilobase pair fragments are derived from the most abundant variant *Pst* segment, their display would be accounted for by the probe reacting with partial DNase I fragments from both ends of this 4.0-kilobase pair *Pst* segment. The two slightly longer and more sharply defined minor daughter fragments remain mysterious; they may be due partly to local DNase I sequence preference, shown in the control naked DNA digest (Fig. 3b, lane 5).

DNase I sensitivity of the active hsp 70 sequences

The 5' sensitivity described so far is present before induction. Figure 4a shows the state of hsp 70 chromatin before and after 15 min of heat shock at 35 °C. Essentially the whole of the active hsp 70 coding sequence becomes sensitive to DNase I²⁸, but with a magnitude less than the 5' sensitivity, which is still partially retained (Fig. 4c, lanes 4, 6). Curiously, some of the normally sensitive sites upstream from the hsp 70 5' ends seem to lose definition with heat shock.

As a control, the DNA blot shown in Fig. 4a was immediately reprobbed with another cloned segment not related to the heat shock genes, pDm4 (ref. 29), locus 62F; the previous label was not removed. Densitometry (Fig. 4c), of the resulting blot in Fig. 4b shows that all hsp 70 restriction fragments are selectively

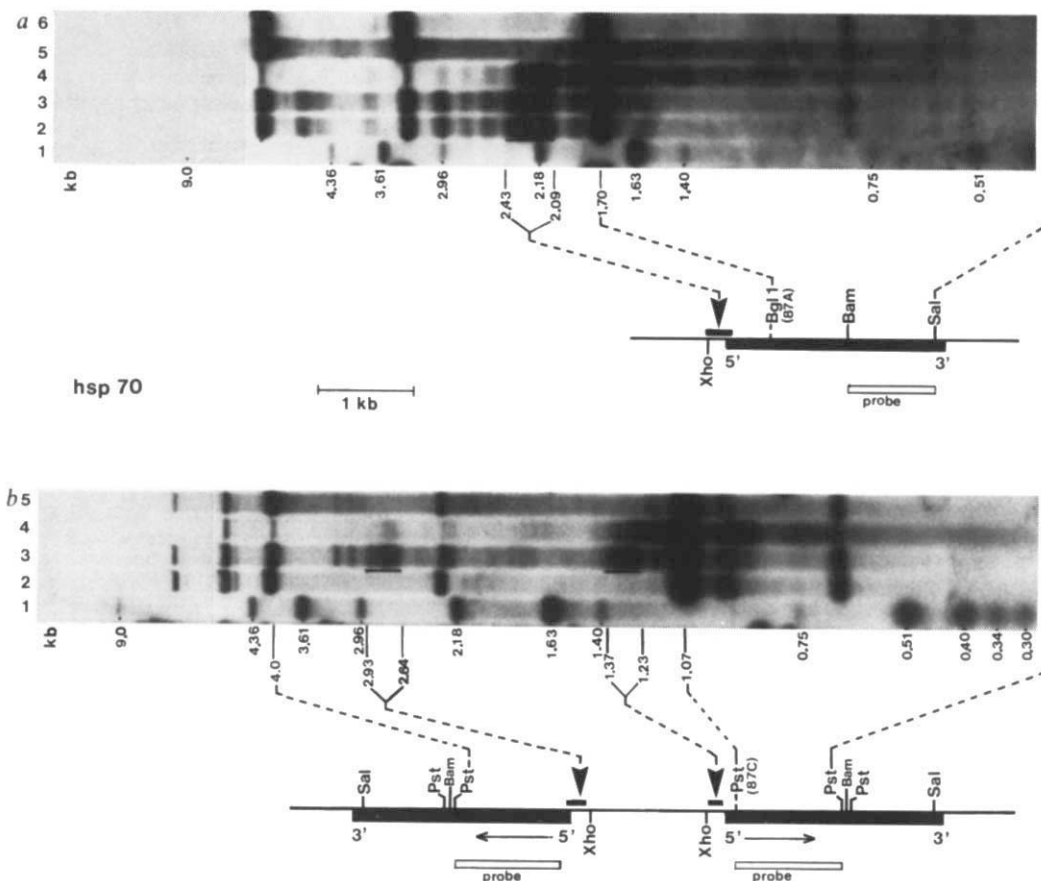
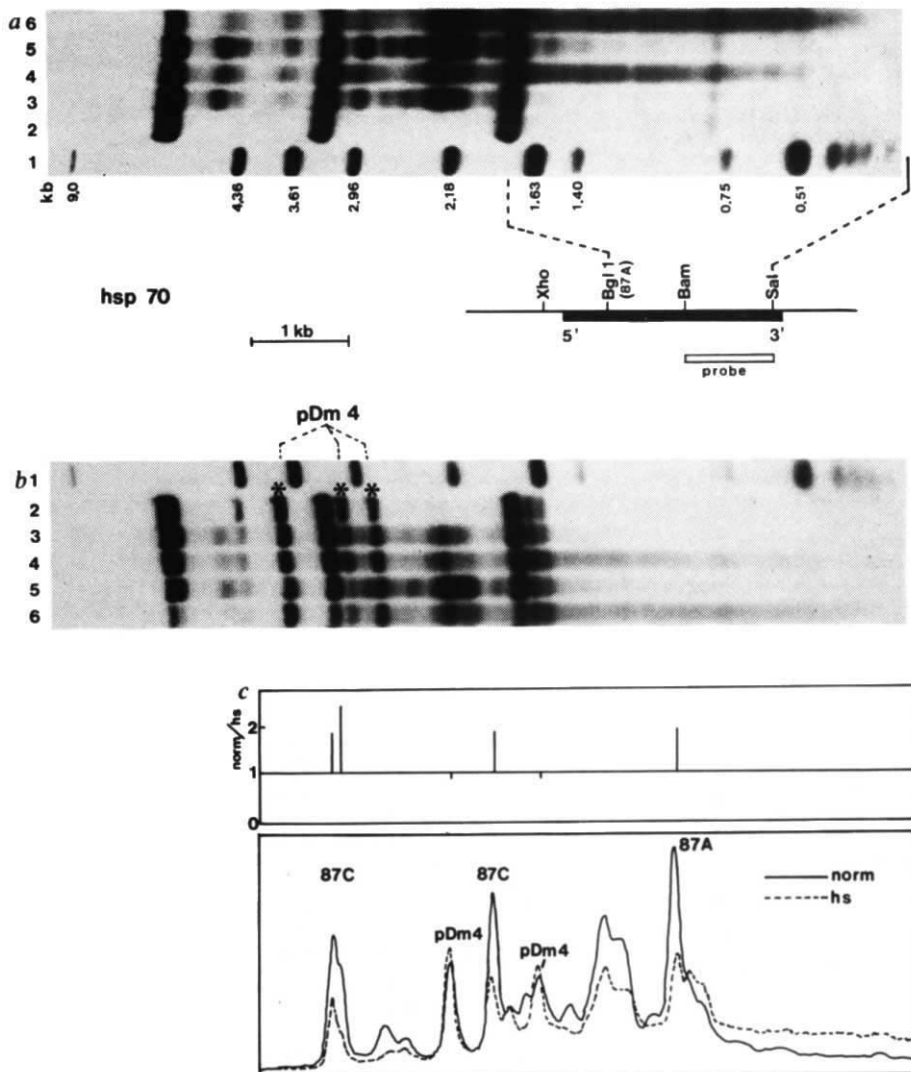


Fig. 3 a, Autoradiogram showing 87C locus-specific DNase I cuts in Schneider cell chromatin upstream from the hsp 70 3' *Sal* site. *Sal*I and *Bgl*I were used for the secondary cleavage; the DNA fragments were sized on a 1.2% agarose gel; pPW229.1 (ref. 17) was the labelled probe. The lanes indicate: 1, Marker DNA fragments. 6, *Sal* plus *Bgl*I digest on Schneider cell DNA. 2–4, Partial DNase I digests on chromatin from two separate experiments, using a final DNase I concentration of 8 units ml^{-1} , and 2 and 4 units ml^{-1} , respectively. 5, Partial DNase I digest on naked DNA, as in Fig. 1a, lane 7. b, Autoradiogram showing 87A locus-specific DNase I cuts in Schneider cell chromatin upstream from a central *Pst* site in the hsp 70 coding region. *Pst*I was used for secondary cleavage; the DNA fragments were sized on a 1.3% agarose gel, and pPW232.1 (ref. 17) was the labelled probe. Only the restriction map for the 4.0-kilobase pair *Pst* fragment is shown, redrawn from the map of clone 122, from ref. 25. See text for discussion. The lanes indicate: 1, Marker DNA fragments. 2, *Pst* digest on Schneider cell DNA. 3, 4, Partial DNase I digests on chromatin at a final DNase I concentration of 2 and 4 units ml^{-1} , respectively. 5, Partial DNase I digest on naked DNA, as in Fig. 1a, lane 7.

Fig. 4 *a*, Autoradiogram showing the state of hsp 70 chromatin before and after heat shock induction. *Sal*I and *Bgl*I were used for secondary cleavage; the DNA fragments were sized in a 0.8% agarose gel, and pPW229.1 (ref. 17) was the labelled probe. The lanes indicate: 1, Marker DNA fragments. 2, *Sal* plus *Bgl*I digest on Schneider cell DNA. DNA from heat-induced cells gave an identical pattern (data not shown). 3, 5, Partial DNase I digests on uninduced Schneider cell chromatin at a final DNase I concentration of 6 and 8 units ml⁻¹, respectively. 4, 6, Partial DNase I digests on chromatin in Schneider cells heat shocked at 35 °C for 15 min, as described elsewhere²⁸. The two digestion points (final DNase I concentrations of 2 and 4 units ml⁻¹, respectively) were chosen out of a series for comparison against those in lanes 3 and 5 because of their close equivalence to the latter in overall extents of DNase I cleavage. They were monitored by gel electrophoresis and ethidium bromide staining (data not shown) and by reprobing this DNA blot with a cloned *Drosophila* DNA segment not involved in the heat shock response (see *b*). *b*, Autoradiogram showing the state of the chromatin at sequences homologous to pDm4 (ref. 29) before and after heat shock induction. The DNA blot in *a* was immediately reprobbed with labelled pDm4 without removal of the previous label. See legend to *a* for key to the lanes. Both pDm4- and pPW229.1-specific fragments are displayed. The most prominent fragments labelled by pDm4 are indicated by asterisks. *c*, A densitometric scan of lanes 5 and 6 in *b*. The ratios of the peak heights between normal (norm) and heat shocked (hs) conditions for the pPW229.1- and two pDm4-specific fragments are schematically displayed over the scan.



digested after heat shock, whereas the pDm4 fragments are not. This indicates that most or all of the five gene copies of hsp 70 are in the active chromatin conformation, consistent with the finding that transcription occurs at both 87A and 87C loci after heat shock^{17,24}.

Hsp 83 chromatin

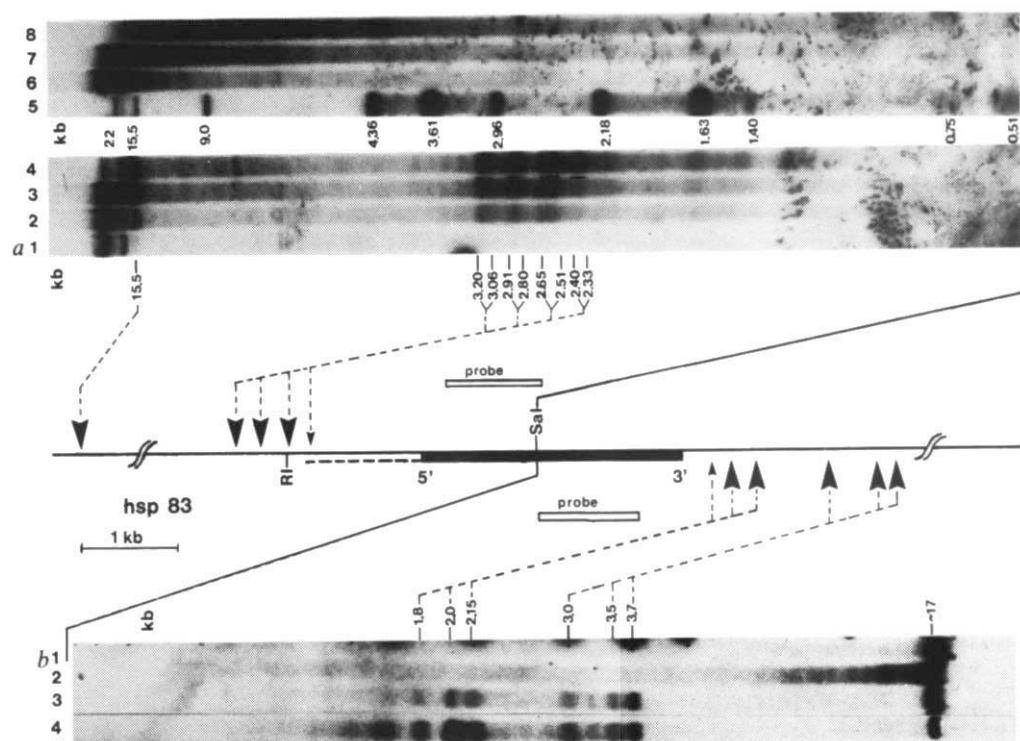
The sequences for the 83,000 dalton heat shock protein, hsp 83, reside at the cytogenetic locus 63BC (ref. 24). From a *Sal*I restriction site within the single-copy coding sequence²⁴, partial DNase I cleavages upstream and downstream in a region extending about 40 kilobase pairs were mapped (Fig. 5*a, b*). A cluster of preferred cuts lie about 2.3–3.2 kilobase pairs upstream from the internal *Sal* site, and two clusters are observed starting from about 1.8 kilobase pairs downstream. No preferred cuts lie near the internal *Sal* site (data not shown). Holmgren and Meselson (manuscript in preparation) have recently mapped the 5' terminus of a precursor to the hsp 83 mRNA to about 160 base pairs downstream from the *Eco*RI site indicated in Fig. 5. Thus, a DNase I-hypersensitive region overlaps or is very near the sequences complementary to the 5' end of this RNA. No known genes occur at the clusters of preferred cuts downstream from the hsp 83 sequence. A low level of hsp 83 RNA is produced in normal conditions in Schneider cells (R. Holmgren and M. Meselson, in preparation). This would account for the slight sensitivity to DNase I shown by sequences homologous to RNA in Fig. 5. Previous single DNase I digests at this locus show that at 35 °C these sequences become more DNase I sensitive³⁰.

Nucleosome arrangement near DNase I-sensitive regions

The basic structural unit of eukaryotic chromatin is the nucleosome, composed of about 200 base pairs of DNA, of which 140 base pairs is wrapped around a core of histones (see ref. 1 for review). Analysis of the nucleosome organization of the hsp 70 genes has been complicated by the multiplicity of the gene copies. In Fig. 6, I map the partial micrococcal nuclease cuts upstream from the *Sal* site in the single-copy hsp 83 coding region; this nuclease preferentially cleaves the linker or spacer DNA between nucleosome cores.

The specific cuts made by micrococcal nuclease in chromatin (Fig. 6, lanes 2–5) reveal a striking degree of order in nucleosomal arrangement, in spite of the sequence specificity shown by the enzyme on naked DNA³¹ (Fig. 6, lanes 7–9). Each of the three DNase I-hypersensitive sites near the 5' end of the gene for hsp 83 is flanked by distinctive micrococcal nuclease cuts. The relative intensity and 100–110-base pair interval between the flanking cuts suggest a deviant structure at these regions. Further upstream, eight specific cuts are evident, beyond which nonlinear mobility of the DNA fragments and special sensitivity of some sequences make interpretation of the pattern difficult. However, the data show sufficiently that those eight nucleosomes are positioned specifically on the DNA^{32–35} (but see ref. 36), and furthermore, that the spacing between adjacent nucleosome cores is irregular (suggested from previous general studies; see ref. 1), determined probably by the local DNA sequence³⁴. Downstream from the region of DNase I

Fig. 5 a, Autoradiogram showing the partial DNase I cuts in Schneider cell chromatin upstream from the *Sal* site in the hsp 83 coding region (redrawn from Holmgren *et al.*²⁴). The position of the *EcoRI* site was determined on a DNA blot not shown here. The filled bar depicts the location of the coding region downstream from the single intervening sequence in this split gene (R. Holmgren and M. Meselson, in preparation). *Sal*I was used for secondary cleavage; the DNA fragments (10 µg per track) were sized on a 1% agarose gel, and the labelled probe was a subclone from pPW244 (ref. 24) containing the *Pst*I segment representing approximately the 5' half of the hsp 83 coding region. This probe extends through the internal *Sal* site by about 50 base pairs (see restriction map) but the small extra segment barely, if at all, labels the cleavages downstream. The lanes indicate: 1, *Sal*I digest on Schneider cell DNA. A minor polymorphism at the external *Sal* site, or cross-reaction with a partially homologous fragment is observed. 2–4, Partial DNase I digests on chromatin at a final DNase I concentration of 0.5, 1 and 2 units ml⁻¹, respectively. Only the more prominent cleavages are noted by the arrows on the restriction map. 5, Marker DNA fragments. 6–8, Partial DNase I digests on naked DNA. DNase I at a concentration of 0.025, 0.05 and 0.1 unit ml⁻¹, respectively, was reacted with the DNA at 0.4 mg ml⁻¹ in digestion buffer for 1 min at 25 °C. **b**, Autoradiogram showing the partial DNase I cuts in Schneider cell chromatin downstream from the *Sal* site in the hsp 83 coding region²⁴. *Sal*I was used for secondary cleavage; the DNA fragments were sized on a 0.8% agarose gel; the cloned probe was a *Sal*-*Hae*III fragment gel-purified after cleavage from pPW244, representing approximately the 3' half of the hsp 83 coding region²⁴. A Du Pont Lightning Plus intensifying screen was used for this film exposure. The lanes indicate: 1, *Sal*I digest of Schneider cell DNA. 2, Partial DNase I digest of naked DNA, as in **a**, lane 6. 3, 4, Partial DNase I digests on chromatin, at a final DNase I concentration of 6 and 8 units ml⁻¹, respectively. This experiment was separate from that in **a**, lanes 3, 4, but the overall extents of cleavage are comparable. Only the more prominent cleavages are indicated by arrows on the restriction map.



hypersensitivity, the micrococcal nuclease cuts are less discrete, except for a cut 1.2 kilobase pairs from the *Sal* reference site. Such disperse cuts^{28,30} would be expected for the slightly active hsp 83 gene at 25 °C. On further activation at 35 °C, the fragment pattern in Fig. 6 remains essentially similar (data not shown).

Comparison of hypersensitivity in cultured cells and embryos

Figure 7 shows a comparison of hypersensitive sites in chromatin from Schneider line 2 cells and from 6–18-h *D. melanogaster* (Oregon R) embryos. The 5' sensitivity in the hsp 70 and hsp 83 genes (denoted by asterisks in Fig. 7a, b) is essentially the same

for both materials, in spite of the populational polymorphism in external restriction sites shown largely by the Oregon R DNA. Intriguingly, several randomly selected regions not involved in the heat shock response [pDm4 (ref. 29), locus 62F, and pPW100 (ref. 2), loci 57BC, 83C] reveal sensitive sites (denoted by asterisks in Fig. 7c, d) in the cell line, but not in Oregon R embryos. This result suggests that some hypersensitive sites in chromatin are not omnipresent, but perhaps limited to certain cell types or to particular stages in development.

An element for recognition?

The two *Drosophila* genes examined at three chromosomal loci exhibit a DNase I-sensitive chromatin structure at the 5'

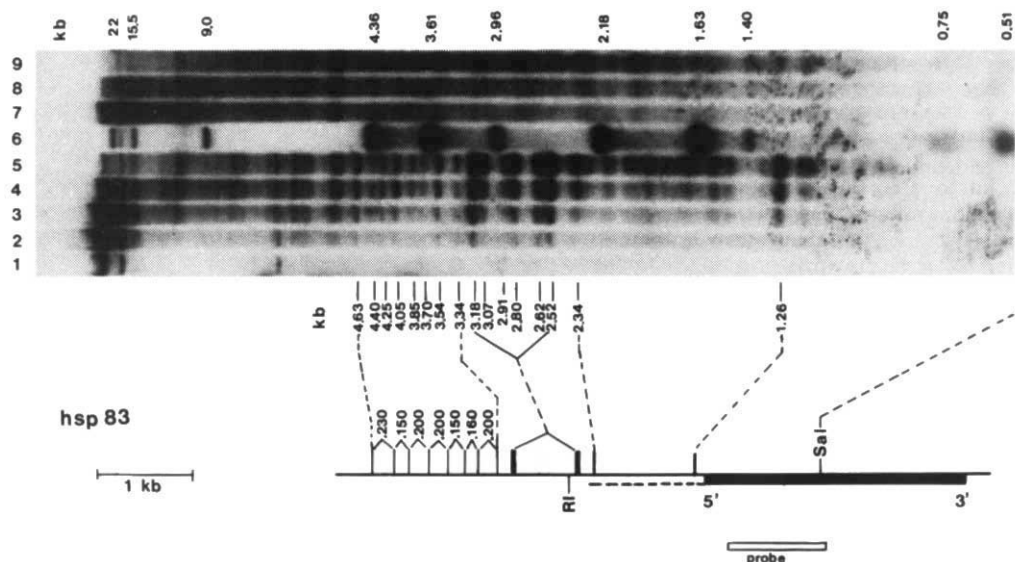
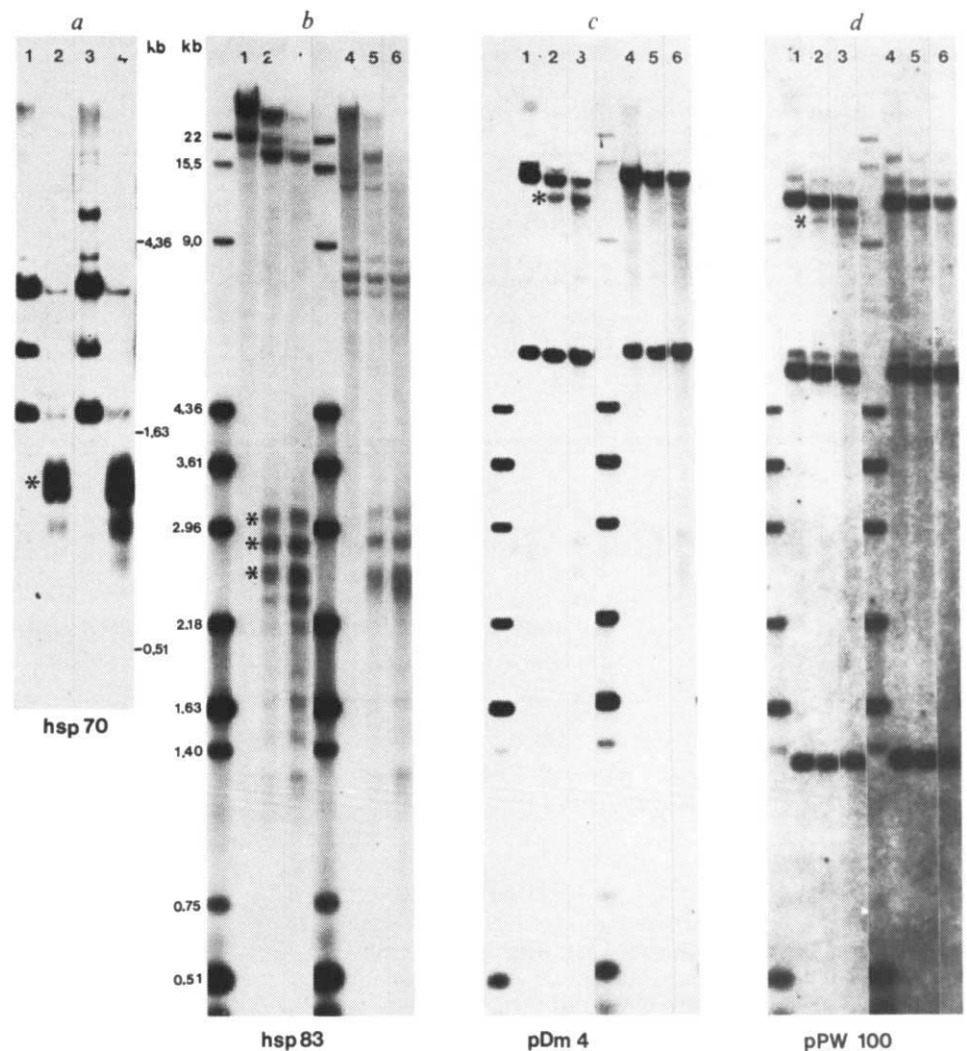


Fig. 6 Autoradiogram showing the partial micrococcal nuclease cuts in Schneider cell chromatin upstream from the *Sal* site in the hsp 83 coding region; produced as in Fig. 5a. The lanes indicate: 1, *Sal*I digest on Schneider cell DNA. 2–5, Partial micrococcal nuclease (Worthington) digests on chromatin at a final nuclease concentration of 0.75, 1.5, 3.0, 6.0 units ml⁻¹, respectively, at 25 °C for 3 min. 6, Marker DNA fragments. 7–9, Partial micrococcal nuclease digests on naked DNA at a final nuclease concentration of 1.25, 2.5 and 5 units ml⁻¹, respectively, at 25 °C for 1 min.

Fig. 7 Autoradiographic comparison of DNase I-hypersensitive sites at chromosomal regions homologous to four cloned segments, between Oregon R embryos and Schneider line 2 cells. Nuclei from 6–18-h embryos were isolated as described elsewhere². The rest of the experiment was essentially as described in Fig. 1 legend. *a*, The hsp 70 region. DNA fragments were sized on a 1.1% agarose gel; *Bam*HI was used for secondary cleavage and the blot was probed with pPW232.1 (ref. 17). The lanes indicate: 1, 3, *Bam*HI digest of Schneider cell and Oregon R DNA, respectively. 2, 4, Partial DNase I digests of Schneider cell (8 units ml⁻¹) and Oregon R (6 units ml⁻¹) chromatin, respectively. *b*, The hsp 83 region. DNA fragments were sized on a 0.8% agarose gel; *Sal*I was used for secondary cleavage; the blot was probed as in Fig. 5*a*. The lanes indicate: 1, 4, *Sal*I digest of Schneider cell and Oregon R DNA respectively. 2, 3, Partial DNase I digests (6 and 8 units ml⁻¹, respectively) of Schneider cell chromatin. 5, 6, Partial DNase I digests (2 units ml⁻¹ in two separate experiments) of Oregon R chromatin. *c*, *b* was reprobed with labelled pDm4 (ref. 34), without removal of the previous label. *d*, *c* was reprobed with labelled pPW100 (ref. 2), without removal of the previous labels.



terminus of the coding sequence before activation. This 5' sensitivity may reflect a specially exposed or structurally modified stretch of eukaryotic DNA for recognition by regulatory molecules. The other sites adjacent to the hsp coding regions may be indicative of the 5' termini of unknown or yet unmapped transcription units, as in the repeated sequence elements at locus 87C (refs 17, 27), or they may reflect other chromosomal functions. The findings documented here, although unique to eukaryotic gene structure, have a precedent in the structure of viral chromatin; the origin of replication (and transcription) of SV40 and polyoma virus has been shown to be in a preferentially nuclease-susceptible conformation^{37–39}.

What might be the basis of this unique sensitivity? A special DNA sequence at these regions could bind other macromolecules such as specific proteins that cause the sensitivity and align neighbouring nucleosomes specifically on the DNA, or the sequence may possess a peculiar base order that renders it sensitive when the DNA in the region is coiled and supercoiled in nucleosomes and higher order structures. The molecular basis notwithstanding, a new regulatory feature in relation to prokaryotic models of gene control appears: an element for recognition at the 5' end of a gene based on an exposed chromatin structure that defines its potential for activation, this potential being realized on induction, perhaps according to the bacterial paradigm of specific activators and repressors. Such a two-stage model of eukaryotic gene regulation has some implications for developmental biology. Specifically, the hypersensitive sites observed in tissue culture cell chromatin but not in embryonic chromatin might suggest that a developmentally regulated gene (as opposed to the heat shock genes) possess a 5' exposed structure only in the appropriate cell type or developmental

stage. Sites of DNase I hypersensitivity have recently been observed in the chicken⁴⁰, and Weintraub and colleagues (personal communication) have detected tissue-specific hypersensitive sites for the chick globin gene. Will the various developmental systems currently amenable to molecular analysis yield data consistent with this view of gene regulation?

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Nucleotide sequence of the yeast plasmid

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The nucleotide sequence of the yeast DNA plasmid (2 μ circle) from Saccharomyces cerevisiae strain A364A D5 has been determined. The plasmid contains 6,318 base pairs, including two identical inverted repeats of 599 base pairs. Possible functions are suggested, and attributes of an improved vector for cloning foreign DNAs in yeast are discussed.

THE yeast plasmid (2 μ circle) is a circular DNA molecule with a contour length of about 2 μ m (about 6 kilobases) that is present in about 70 copies per cell of most yeast strains and constitutes about 3% of the total yeast cellular DNA^{1–3}. Its most distinguishing structural feature is the presence of two inverted repeat (IR) sequences of about 600 base pairs that are separated by a large unique region (about 2.7 kilobases) and a small unique region (about 2.3 kilobases)^{3–8}. In yeast cells reciprocal recombination between the inverted repeats produces a mixture of two sequence forms (A and B) of the yeast plasmid that differ in the orientation of one unique region relative to the other^{3–9}. When the yeast plasmid is cloned in *Escherichia coli* using recombinant DNA techniques this reciprocal recombination does not occur.

Two observations suggest that the yeast plasmid is located, at least some of the time, in the yeast nucleus. First, the plasmid can be isolated in a condensed nucleosome form with which histones are associated, similar to yeast nuclear chromatin^{10,11}. Second, its replication occurs only during the S phase, each molecule is replicated only once, and replication is regulated by the same genes that control the initiation and completion of nuclear DNA replication^{12,13}. There is no evidence that the yeast plasmid is capable of integrating into chromosomal DNA.

The biological function of the yeast plasmid is not known. However, portions of the plasmid are transcribed *in vivo* into polyadenylated mRNAs which have been mapped relative to restriction enzyme fragments of the DNA¹⁴. The poly(A)-containing RNAs direct the synthesis of protein in *in vitro* translation experiments. Plasmid-specific proteins have been detected when the plasmid has been introduced into *E. coli* minicell-producing strains^{3,15,16}. Yeast strains have been isolated which do not have any plasmid sequences present, but possess normal growth properties in laboratory conditions^{17,18}.

Recently the yeast plasmid has acquired increased significance as an efficient vector for yeast transformation^{19–24}. Experiments with chimaeric DNAs containing all or part of the yeast plasmid indicate that maximum transformation stability is obtained when one of the two inverted repeats is present²⁵, suggesting that the replication origin of the plasmid may be within this sequence. Thus the yeast plasmid is an attractive

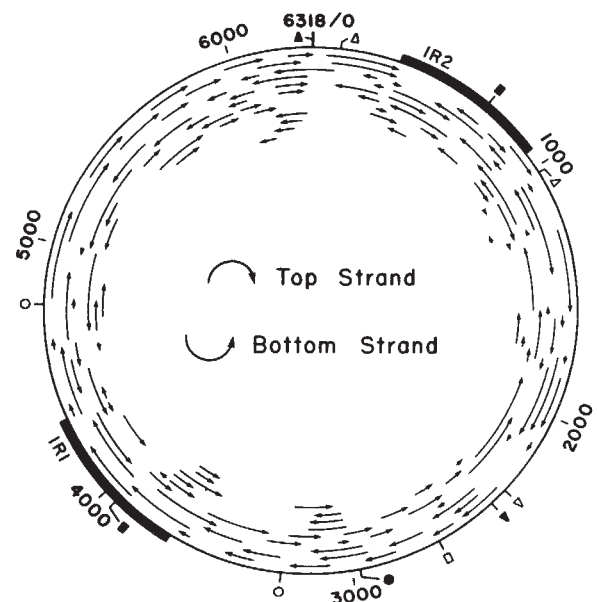


Fig. 1 Map of the yeast plasmid (form A) and the sub-sequences used to derive the sequence. Arrows indicate the location and extent of each subsequence, clockwise for the top strand, counter-clockwise for the bottom strand. Thick lines represent the inverted repeats IR1 and IR2. Restriction enzyme cleavage sites shown are *EcoRI* (Δ), *HindIII* (∇), *PstI* ($\square$), *HpaI* ($\bullet$) and *AuaI* ($\circ$).

system for the study of eukaryotic gene control and chromosomal replication.

We report here the sequence of the yeast plasmid. The boundaries of the inverted repeats are defined and potential protein coding regions are identified. The location of the origin of replication and the function of one of the protein coding regions are discussed. Improvements in the cloning of foreign DNAs in yeast are suggested.

Source of the sequence

For the sequence determinations, the yeast plasmid was recovered from the recombinant plasmid p82-6B. This is a plasmid which was found in a library of random yeast strain A364A D5 DNA fragments cloned in the *E. coli* plasmid pMB9 (ref. 26) by the poly(dA)-poly(T) tailing technique²⁷. It was initially identified by screening individual colonies in the library for the presence of yeast plasmid sequences by the Grunstein and Hogness procedure²⁸ and subsequently shown to contain about 1.5 copies of the yeast plasmid (E. Gubbins, K. Chen and J.E.D., unpublished data). The 1.5 copies probably arose from a yeast plasmid dimer that was tailed with poly(dA) at random internal single-strand nicks or double-strand scissions during the poly(dA) addition such that part of the dimeric sequence was removed during the cloning procedure.

The circular yeast plasmid (form A) contains two *EcoRI* sites, one in each of the two unique regions, such that *EcoRI* digestion generates two fragments of 3.9 and 2.4 kilobases (ref. 5). The initial sequencing strategy was to determine the sequence of these two fragments individually and then identify and sequence restriction fragments which would overlap the two *EcoRI* sites. As the sequencing progressed, it became convenient to subclone a 4.1 kilobase *HindIII* fragment that contained the entire 3.9-kilobase *EcoRI* fragment and hence possessed an *EcoRI* site near each end. Thus the sequence of the 2.4-kilobase *EcoRI* fragment was obtained using the fragment recovered directly from p82-6B, and the rest of the sequence was largely obtained from the cloned 4.1-kilobase *HindIII* fragment subcloned in pBR322. Both plasmids were completely stable when maintained in the *recA E. coli* strain HB101.

The sequence of the plasmid was obtained by collecting sequence data from as many 3'-terminally labelled *HpaII*-, *Sau3AI*-, *TaqI*-, and *HinfI*-fragments as could be strand separated²⁹ or digested with a second restriction enzyme to separate the labelled ends. In practice, about three-quarters of the fragments could be separated into two distinct strands on a strand separation gel. Overlapping sub-sequences were identified by computer analysis³⁰ or by inspection. Restriction sites found during this process were used for subsequent sequencing runs to fill gaps or to increase coverage of both strands. The individual sequences (about 140 in all) were each read at least three times. All changes and corrections were made on a computer file to minimize clerical error.

Features of the sequence

Figure 1 shows a map of the restriction fragments used to obtain the sequence data and Fig. 2 shows the complete sequence. The inverted repeats are designated IR1 and IR2 according to the nomenclature of Hollenberg¹⁶. Of the 6,318 base pairs, 61% are dA+dT, close to the dA+dT content of yeast chromosomal DNA (about 64%) and much lower than the value for mitochondrial DNA of 79% (ref. 3). The sequence is numbered taking nucleotide 1 as the G of the *EcoRI* site nearest IR2. The inverted repeats are 599 base pairs long, extending from 341 through to 939 (IR2) and from 3,714 to 4,312 (IR1). The unique regions are therefore 2,774 and 2,346 base pairs long. The sequence contains previously mapped sites for the restriction enzymes *EcoRI* (1; 2,407), *PstI* (2,652), *HindIII* (105; 1,017; 2,331), *HincII* (217; 2,964), *HpaI* (2,964; subset of *HincII* sites), *AvaI* (3,258; 4,764) and *XbaI* (703; 3,945). In addition, sites for *PvuI* (1,755; 2,729), *PvuII* (2,029; 2,133), *BalI* (264), *BclI* (304) and *ClaI* (6,284) are predicted. Sites for the enzymes *BamHI*, *KpnI*, *SalI*, *SstI*, *SstII*, *XhoI*, *BglI* and *BglII* do not appear in the sequence. A total of 87% of the plasmid was sequenced on both strands and at least one overlap was obtained for all restriction sites used in sequencing. The sequence of the region 1-1,022 has been reported previously by another laboratory³² and differs from that presented here at eight positions.

The inverted repeats are the most prominent feature of the plasmid. Their sequences were determined separately and found

to be completely homologous. The IR boundaries are not marked by any striking symmetry features. However, a remarkable concentration of symmetry elements occurs near the centres of the IRs, at 640-761 and 3,892-4,013. In these 122-base pair regions are found a 13-base pair direct repeat and a dyad symmetry (abc-c'b'a') of 16 base pairs, both contained within an imperfect dyad symmetry of 49 base pairs. These regions can be drawn as two alternative stem and loop structures (Fig. 3) which would have thermodynamic stabilities of the order of $-50 \text{ kcal mol}^{-1}$ when calculated by rules derived from oligoribonucleotide studies³³. The longest run of alternating purines/pyrimidines found in the plasmid is located at 564-581 and 4,072-4,089. It has been proposed that these might form left-handed helical structures *in vivo*³⁴. Possible implications for these features are discussed below.

A second concentration of symmetry elements is found in the large unique region. About 5.5 copies of a sequence of 62-63 base pairs arranged as direct tandem repeats are found between positions 2,960 and 3,302 (Fig. 2). Five repeats, 2,987-3,048, 3,049-3,111, 3,112-3,173, 3,174-3,235 and 3,236-3,297, share 78-97% (average 90%) homology with a consensus sequence

5'-TTTTTPuPyAGAACAAAAATGCAACGCGAGAGCG
CTAATTTT $\frac{A}{T}$ CAAACAAAGAATCTGAGCTTCA-3'.

Two sets of two of these repeats, 2,975-3,098 and 3,099-3,223, can be aligned to give tandem copies of 124 base pairs which share 97% homology. In addition, this area is rich in dyad symmetries, including a perfect dyad at 2,987-3,002 and 3,236-3,251. The significance of these symmetries is not known. They appear to be dispensable to the plasmid, however, since deletions in this area are seen in natural isolates⁵. No large alphabetic palindromes (abc-cba) were found in the yeast plasmid.

The sequence was examined for potential protein-coding regions, that is, regions starting with an ATG codon and terminating with any of the stop codons. Three such long open translation reading frames were found, 'Able' (423 codons, molecular weight (MW) 48,625; 5,570-520; top strand; reading frame 2), 'Baker' (373 codons, 43,235 MW; 2,008-890; bottom strand; reading frame 3) and 'Charlie' (296 codons, MW 33,199; 5,198-4,311; bottom strand; reading frame 2) as shown in Fig. 4. All three coding regions terminate within inverted repeats, although 'Baker' ends just inside the IR1 boundary. 'Able' terminates with TAA, 'Baker' with TAG and 'Charlie' with TGA. Proteins of MW 20,000 and less could be encoded by other regions. Overlapping coding regions such as are seen in several circular viral genomes³⁵⁻³⁷ are not found in the yeast plasmid.

Homology between the yeast plasmid and the bacterial cloning vector pBR322 has been reported³⁸ on the basis of hybridization experiments. Computer comparisons of the two DNAs under various homology requirements did not reveal any extensive identities. The longest perfect homology is 13 base pairs.

Discussion

The sequence of the yeast plasmid can be inspected from the standpoint of (1) its utilization as a vector for cloning foreign DNA fragments, and (2) its existence as a eukaryotic 'mini-chromosome' organized by histones into nucleosomes, and containing an origin of replication and one or more genes with attendant regulatory elements. Two conclusions regarding the plasmid's *in vivo* functions can be drawn from the sequence and from the work of other investigators: the location of the origin of replication and the identification of the gene product which mediates the interconversion of forms A and B. Both conclusions concern the use of the yeast plasmid as a cloning vehicle.

Several lines of evidence support the suggestion that the origin of replication of the yeast plasmid is near the *XbaI* site in IR1 (position 3,945). First, the regions surrounding the *XbaI*

10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
GAATTCGAA	CCAGGCTTAA	AACGAGTAA	TAGGACGBC	AATTCCTCAA	GCAATAAACA	GGAATACCAA	TTATTAATTA	ATACTTAGT	CAGATCGAT	AATAAAGCT	TGAAGAACT	TGCGCCATT	TCATCTTCT	CTATAAATA
CTTAGAGAT	GGTCAGGAT	TGCTCATAT	ATCCGBCGC	TTAAGAGAT	CGTTATTGT	CGTTATTGT	AAATATTTC	TATTAATTA	CTTAGAGAT	TAATTCGAA	ACTCTTTTT	ACGCGGATA	AGTTAGAAAC	GATATTTTTT
160	170	180	190	200	210	220	230	240	250	260	270	280	290	300
TGGCCCAAAA	TTCTACATT	BAAGACAT	GATGACCTA	TTTCTTCAA	TGAAGGCGT	ACGAGGATG	ACTAATGTT	TGGAAGATG	GAGCAGATG	CGCTGCTCT	CGGTGBCAG	GACAAGCTA	ACTCAGCBA	TAAACGAA
ACCGGGTTTT	AGAGTGAAC	CTTCTGAAA	CTAGCGGAT	AAAGAAAGT	ACTTCCGGA	TGBCCTCAAC	TGATACCAAC	ACCTCTTAC	CTGCTATTC	GCACGAGAC	GCACCGGCT	CTGTGTCAT	TGATAGTCT	ATTGTGCTA
310	320	330	340	350	360	370	380	390	400	410	420	430	440	450
ACCTGATCAG	TACTTCGAC	TAGTTTCTC	GTACTATGCA	TATGATCCAA	TATCAAGGAA	AATGATGACA	TGGAAGATG	ABACTAATC	AATTBAGGAG	TGBCACGAT	TAGAACAGTA	AAAGGGTAGT	GCTGAGAGCA	GCATAGCTA
TGACATAGT	ATGAGCGTG	ATCAAGAGC	CATGATGAT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT	ATAGTTTCT
460	470	480	490	500	510	520	530	540	550	560	570	580	590	600
CGGCGATAG	AATGAGTAA	TATGAGTAA	BTACTAGTAA	TAGCTTTAT	CTCATATAA	TAGACGATA	TAGATGACA	TTTAAGATA	AACAGCAGT	ATGCGCTCT	TCTATGAT	ATATATATA	AGGCAACAG	CAGATATAG
GGGCGTACC	TACCTCAT	ATGATGCT	CCATGATG	ATGGAAGTA	GGATGATAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT	ATCTGCTAT
610	620	630	640	650	660	670	680	690	700	710	720	730	740	750
TGCGATGTA	ACAGTGAAT	GTATGCGC	AGCTGCGCT	GCATTTTCT	AAAGCTGCT	TTTCCGAA	CGTTTGAAT	TCCTTCTCT	AAATTTCTT	AAATTTCTT	AAATTTCTT	AAATTTCTT	AAATTTCTT	AAATTTCTT
ACGCTGATC	TGCTACGTA	CATACGCG	TGAGGCGCA	CGTAAAGCC	TTTCCGAA	AAAGCTTCT	CGAACTTCT	CGAACTTCT	CGAACTTCT	CGAACTTCT	CGAACTTCT	CGAACTTCT	CGAACTTCT	CGAACTTCT
760	770	780	790	800	810	820	830	840	850	860	870	880	890	900
GAATTCGAA	CCAGGCTTAA	AACGAGTAA	TAGGACGBC	AATTCCTCAA	GCAATAAACA	GGAATACCAA	TTATTAATTA	ATACTTAGT	CAGATCGAT	AATAAAGCT	TGAAGAACT	TGCGCCATT	TCATCTTCT	CTATAAATA
CTTAGAGAT	GGTCAGGAT	TGCTCATAT	ATCCGBCGC	TTAAGAGAT	CGTTATTGT	CGTTATTGT	AAATATTTC	TATTAATTA	CTTAGAGAT	TAATTCGAA	ACTCTTTTT	ACGCGGATA	AGTTAGAAAC	GATATTTTTT
1060	1070	1080	1090	1100	1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
TACCGGATA	ACCTTTAGC	GTTCCTGAG	ATGATTAAT	TTCTTTATG	ATATGATCA	ABACACTTT	GAATTTTGA	CGACGAAAT	TGAGTTTCT	CATCTCTCT	CTATTCTCA	TTATCTTCT	GGCTATTAT	TGCGGCTAG
ATGCGCTAT	TGGAATATG	CAGGAGACT	TACCTTATC	AAGGAATCA	TATACTATG	TCTGTGAAA	CTTGAAGAA	CGCTCTTAA	ATCTTCTAA	ATCTTCTAA	ATCTTCTAA	ATCTTCTAA	ATCTTCTAA	ATCTTCTAA
1210	1220	1230	1240	1250	1260	1270	1280	1290	1300	1310	1320	1330	1340	1350
TTGATCTTC	CGCTGACAG	TGCTTTATC	CACATATAT	ATCTATTCT	CCCACTATC	CTTCTGCG	CTTCTGCG	CTTCTGCG	CTTCTGCG	CTTCTGCG	CTTCTGCG	CTTCTGCG	CTTCTGCG	CTTCTGCG
AAACTAGAA	GAAGTATCT	ACAGTAAAC	ATGATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT	AAATTAAT
1360	1370	1380	1390	1400	1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
GTCTGATCT	TCCTATATG	CTTATATCT	GTCTTCTCT	CACAACTAT	GAATTTTGA	CGATTAAT	CGATTAAT	CGATTAAT	CGATTAAT	CGATTAAT	CGATTAAT	CGATTAAT	CGATTAAT	CGATTAAT
CGAGGACTAG	AGGATATAT	GAATATAGA	CAAGAGAAAT	GTGTTGAAT	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA	CTTACATAA
1510	1520	1530	1540	1550	1560	1570	1580	1590	1600	1610	1620	1630	1640	1650
ATTGATAT	ATTGATAT	ATTGATAT	TTTATATAT	ATCTGAGAA	TAGAGAGAA	ATGAGAGAA	CTTGTATAT	ACAAATGAG	ATTCTATAT	TCATTAATAT	ACBCTTATG	GCCTTCTAT	ACCTTTCTC	CTGCGTTAT
TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA	TAATATATA
1660	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
CGCTGCTCT	TACATATA	TCTAACTCT	CTTCTGAGT	GCCTTTTAT	TGACTAATA	ATGCACTAT	TATTAATCT	ATAATTTCT	GTGATTTAT	GAATTTTAT	GAATTTTAT	GAATTTTAT	GAATTTTAT	GAATTTTAT
CGCAGGAAA	ATGATATAT	AGATTTGGA	GAGGCTACCA	CGGAAATTT	ACTGATAT	TACGTTGCT	ATATTTGCA	CTATTAATG	CGATTAATG	CGATTAATG	CGATTAATG	CGATTAATG	CGATTAATG	CGATTAATG
1810	1820	1830	1840	1850	1860	1870	1880	1890	1900	1910	1920	1930	1940	1950
GTCTTTTCT	TAATACGCT	GTGCTGCTC	TATTCAGTAT	ATGCTCAACA	AATGCAATG	CTAAGCTAT	GTATTTCTA	TAATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT
CAGAGAAAAG	TAATACGCT	CAGACAGAG	TATTCAGTAT	ATGCTCAACA	AATGCAATG	CTAAGCTAT	GTATTTCTA	TAATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT	CGATTTGCT
1960	1970	1980	1990	2000	2010	2020	2030	2040	2050	2060	2070	2080	2090	2100
GTGGAAGTG	CTGATATA	CATTGCTTA	TACAGCAAT	CACTCTCTC	CGTATATAT	TTCATTTAT	TTCATTTAT	TTCATTTAT	TTCATTTAT	TTCATTTAT	TTCATTTAT	TTCATTTAT	TTCATTTAT	TTCATTTAT
CAACTTTAC	GAGCTATAT	GTAGCAAT	ATGCTGCTC	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT	GTAGCAAT
2110	2120	2130	2140	2150	2160	2170	2180	2190	2200	2210	2220	2230	2240	2250
AAATTTTCT	TACATATA	CACTGCTAT	CCGCTGCTC	TATTTGAAA	TTCGGAAGA	ATGAGAGAA	CGCTGCTAT	ATTTTCTA	CGATTTGCT	TGATTTGCT	TGATTTGCT	TGATTTGCT	TGATTTGCT	TGATTTGCT
TTTGAAGAG	ATGCTTTAT	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC	GTGCTGCTC
2260	2270	2280	2290	2300	2310	2320	2330	2340	2350	2360	2370	2380	2390	2400
AACTTCAAT	TGCTGCTAT	ATGCTTAT	AAACGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT	AGATGCTAT
TTGGAATAG	ACAGTAACT	TACGGAAT	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA	TTTGTGATA
2410	2420	2430	2440	2450	2460	2470	2480	2490	2500	2510	2520	2530	2540	2550
CACTTGAAT	TCAGGCTAT	ATAGCTAT	CTAGTATAT	CGCTGCTAT	TACGATATAT	TTCGCTGCT	GTCTTTCTC	TTTGAAGAG	CTTCTGCTC	CTTCTGCTC	CTTCTGCTC	CTTCTGCTC	CTTCTGCTC	CTTCTGCTC
GTGAATCTTA	AGTGCCTTA	TATCTATAT	GATCATATG	GGCAGTAT	ATGCTATAT	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC	AGGCTGCTC
2560	2570	2580	2590	2600	2610	2620	2630	2640	2650	2660	2670	2680	2690	2700
TCTATCTTC	CGATGATAT	AAACTGAT	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA	GACCGAGAA
AGATAGAGG	GTACATCAT	TTTGTGATA	TGCTGCTAT	CTTGTGATA	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT
2710	2720	2730	2740	2750	2760	2770	2780	2790	2800	2810	2820	2830	2840	2850
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TATAGCTGT	TGACAGAA	GTCTAATAT	TGCTGCTAT	TTACGCTAT	TGGAATTTT	AACTGCTAT	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
2860	2870	2880	2890	2900	2910	2920	2930	2940	2950	2960	2970	2980	2990	3000
TATGATTTT	GTCTGCTAT	GAATTTTAT	CATCTATAT	ATGCTGCTC	TGGAATTTT	AACTGCTAT	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
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3010	3020	3030	3040	3050	3060	3070	3080	3090	3100	3110	3120	3130	3140	3150
ATACAGCTC	GAGAGGCTA	ATTTTCTAA	CAAGAGCTC	GAGAGGCTA	ATTTTCTAA	CAAGAGCTC	GAGAGGCTA	ATTTTCTAA	CAAGAGCTC	GAGAGGCTA	ATTTTCTAA	CAAGAGCTC	GAGAGGCTA	ATTTTCTAA
TATAGCTGT	CTTGTGATA	TATAGCTGT	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
3160	3170	3180	3190	3200	3210	3220	3230	3240	3250	3260	3270	3280	3290	3300
TCAACAGAA	AATCTGCTC	CTTGTGATA	GAAGAGCTC	TGCAAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC
ATTTTCTAA	TTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
3310	3320	3330	3340	3350	3360	3370	3380	3390	3400	3410	3420	3430	3440	3450
TTTGTGATA	TGCTGCTAT	GAATTTTAT	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
AAAGAGGAA	ACAGGCTC	TATCTGATA	GAGAGCTAT	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC	GAAGAGCTC
3460	3470	3480	3490	3500	3510	3520	3530	3540	3550	3560	3570	3580	3590	3600
AGCTGCTCT	GCATTTTCT	AGATTAAGG	CATCTGCTC	TATATTTCT	ACCGATGCT	ATGCTGCTC	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
TGCGGCTCA	CTAAGAGCT	TTTGTGATA	TGCTGCTAT	ATATAGATA	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT
3610	3620	3630	3640	3650	3660	3670	3680	3690	3700	3710	3720	3730	3740	3750
TATAGCTGT	ATAGAGGAT	TTTGTGATA	CTTGTGATA	TGCTGCTAT	ATATAGATA	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT	TGCTGCTAT
ATATAGCTA	TATCTTTAT	AAATGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
3760	3770	3780	3790	3800	3810	3820	3830	3840	3850	3860	3870	3880	3890	3900
AAAGGCTAT	GGATGCTAT	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA
TTTGTGATA	CTAAGCTAT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT	ATATCTGCT
3910	3920	3930	3940	3950	3960	3970	3980	3990	4000	4010	4020	4030	4040	4050
AGAGGCTAT	TTTGTGATA	AAAGGCTAT	TTTGTGATA	AAAGGCTAT	TTTGTGATA	AAAGGCTAT	TTTGTGATA	AAAGGCTAT	TTTGTGATA	AAAGGCTAT	TTTGTGATA	AAAGGCTAT	TTTGTGATA	AAAGGCTAT
CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA	CTTGTGATA
4060	4070	4080	4090	4100	4110	4120	4130	4140	4150	4160	4170	4180	4190	4200
CACCTATAT	TGCTGCTAT	CTTGTGATA	ATATAGATA	GAAGAGCTC	ATATAGATA	GAAGAGCTC	ATATAGATA	GAAGAGCTC	ATATAGATA	GAAGAGCTC	ATATAGATA	GAAGAGCTC	ATATAGATA	GAAGAGCTC
BTGATATAG	ACGAGAGCT	GATATATAT	TATATGCT	CTTGTGATA	TATATGCT	CTTGTGATA	TATATGCT	CTTGTGATA	TATATGCT	CTTGTGATA	TATATGCT	CTTGTGATA	TATATGCT	CTTGTGATA
4210	4220	4230	4240	4250	4260	4270	4280	4290	4300	4310	4320	4330	4340	4350
GTATGCTAT	GTCTGCTAT	GAAGAGCTC	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA	TATAGGATA
CTATAGATA	CGAGAGCT	CTTGTGATA	ATAT											

5540 5570 5580 5590 5600 5610 5620 5630 5640 5650 5660 5670 5680 5690 5700
 CACAAGTCG GCGCTAAGCA TGCCACAATT TGCTATATTA TGTAAACAC CACCTAAGGT GCTTGTTCGT CAGTTTGTGG AAAGGTTTGA AAGACCTTCA GGTGAGAAAA TAGCATTATG TGCTGCTGAA ACGACGACTT GATTGTGATA ATACAACCTA
 GTGTTCTAGC GCGGATTCGT ACGGTGTGTA ACCATATATA ACATTTTGTG GTGGATTCCA GGAACAAGCA GTCACAACCC TTTCCTAACT TTCTGGAAGT CCACCTCTTT ATCGTAATAC ACGACGACTT GATTGTGATA ATACAACCTA
 5710 5720 5730 5740 5750 5760 5770 5780 5790 5800 5810 5820 5830 5840 5850
 GATTACACAT AACGGAACAG CAATCAAGAG ABCCACATTC ATGACCTATA ATACTATCAT AAGCAATTCG CTGAGTTTCG ATATTGTCAA TAAATCAGTC CAGTTTAAAT ACAAGAGACA AAAAGCAACA ATTCTGGAG CCTCATTA
 CTAATGTGTA TTGCTTGTCT GTAGTCTCTC TCGGTGTAG TACTCGATAT TATGATAGTA TTGCTTAAAG GACTCAAGAC TATAACAGTT ATTTAGTGA GTCAAATTTA TGTCTGCGT TTTTCGTTGT TAAGACCTTC GGAGTAATTT
 5860 5870 5880 5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000
 GAAATGATT CCGCTTGGG AATTACAAAT TATTCCTTAC TATGACAAA AACCATCAAT TGATATCACT GATATTGTAA GTAGTTTSCA ATTACAGTTC GAATCATCGG AAGAAGCAGA TAAGGGAAT AGCCACAGTA AAAAAATGT
 CTTTAACATA GGACGAACCC TTAATGTGTA ATAAGGAATG ATACCTGTTT TTGTAGTTAG ACTATAGTGA CTATAACATT CATCAACAGT TAATGTCAAG CTTAGTAGCC TTCTGCTCT ATTCCCTTTA TCGGTGTCT TTTTITACGA
 6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120 6130 6140 6150
 TAAAGCATT CTAAGTGAGG GTGAAGCAT CTGGGAGTC ACTGAGAAAA TACTAAATTC GTTGTAGTAT ACTCTGAGAT TTACAAAAAC AAAAAGCTTTA TACCAATTC TCCTCTAGC TACTTTCATC AATGTGTGAA GATTACGCA
 ATTTCTGAAA GATTCACTCC CACTTCTGTA GACCTCTAG TGACTCTTT ATGATTTAAG CAAACTCATA TGAAGCTCTA AATGTTTGTG TTTTGTAAAT ATGTTAAGG AGAAGGATCG ATGAAGTAG TTAACACCTT CTAAGTCTCT
 6160 6170 6180 6190 6200 6210 6220 6230 6240 6250 6260 6270 6280 6290 6300
 TATTAGAAC GTTGTGCGA AATCATTTAA ATTAGTCAA AATAGTATC TGGAGTAAAT AATCAAGTGT TTAGTGACAG AGACAAAGAC AAGCGTTAGT AAGCACATAT ACTTCTTTAG CCAAGGAGGT ABGATGATC CACTTGTATA
 ATAATTCTG CAATAGAGCT TTAGTAATTT TAATCAGGTT TTATTCATAG ACCCTCATT TTAGGTGACA AATCACTGTC TCTGTTTGTG TTGCAATCA TCGTGTATA TGAAGAAAT CCGTTCCTCA TCTAGTAGC GTGACATAT
 6310
 TTTGGAATGA TTTTGTAG
 AAACCTACTT AAAAAGCT

Fig. 2 Sequence of the yeast plasmid. Boundaries of IR1 (3,714–4,312) and IR2 (341–939) are shown. Symmetry elements are identified by number: 1, regions which can be drawn as stem and loop structures (Fig. 3); 2, longest dyad symmetries (abc–c'b'a') in the sequence; 3, perfect direct repeats; 4, direct tandem repeats of 62–63 bp, or portions thereof; 5, dyad symmetries associated with these repeats. Sequences were determined by the method of Maxam and Gilbert²⁹. Restriction fragments were labelled using repair synthesis of DNA polymerase I (10 units of enzyme in 100 µl of 70 mM Tris–HCl, pH 8.0, 70 mM NaCl, 10 mM MgCl₂, incubated at 4 °C for 10 min with [α-³²P]dNTP and 5–15 pmol of each restriction fragment). Sequencing gels were 0.4 mm thick, 6% (90 × 30 cm) or 20% (43 × 43 cm) polyacrylamide/urea gels run warm⁴².

sites in both IR1 and IR2 contain extensive elements of symmetry which can be drawn as large secondary structures (Fig. 3). Such structures are highly significant statistically (data not shown) and have been found near the origins of replication of many genomes^{39–41}. Second, J. Broach and J. Hicks (J. Broach, personal communication) have cloned various pieces of the yeast plasmid and shown that vectors which contain IR1 can successfully transform [cir^o] yeast (strains which lack endogenous 2µ circles), whereas vectors containing only IR2 cannot. Third, deletion mapping by Broach and Hicks indicates that about 100 base pairs of the large unique region adjacent to IR1 is an essential part of the origin. Since IR1 and IR2 are precisely identical, the involvement of this unique segment presumably determines which inverted repeat will be used for the origin. Examination of translation reading frames of the plasmid (Fig. 4) shows that this is the only segment next to an inverted repeat that is not part of a potential protein coding region. It is possible that this segment (from around position 3,600 to within IR1) is transcribed to yield the RNA primer for initiation of replication. Since electron microscopic examination of replicating yeast plasmid molecules suggests that a second origin, presumably

within IR2, is used at least some of the time (C. Newlon, personal communication), this primer may contain some inverted repeat component.

The yeast plasmid sequence contains three regions which could code for proteins of MWs 33,000, 43,000 and 48,000 (Fig. 4). The location of these are in general agreement with the results of previous studies^{14–16}. Observations of Guerinéau and co-workers^{22,23} suggest a function for one of these regions. They constructed vectors containing the entire yeast plasmid in which the coding region 'Able' was interrupted by the insertion of a piece of bacterial DNA. Such vectors successfully transformed [cir^o] yeast, but no recombination between the inverted repeats was seen, that is, cells transformed with form A vectors maintained the chimaeric DNAs as form A for long periods²³. In contrast, [cir⁺] strains (which contain endogenous yeast plasmid) rapidly interconverted forms A and B, such that only a mixture of the two forms was detected²². Broach and Hicks have made similar observations (J. Broach, personal communication). A diffusible product of coding region 'Able' is thus implicated as mediating the form interconversion process. This gene product and its substrate, the inverted repeats of the 2µ circle, represent

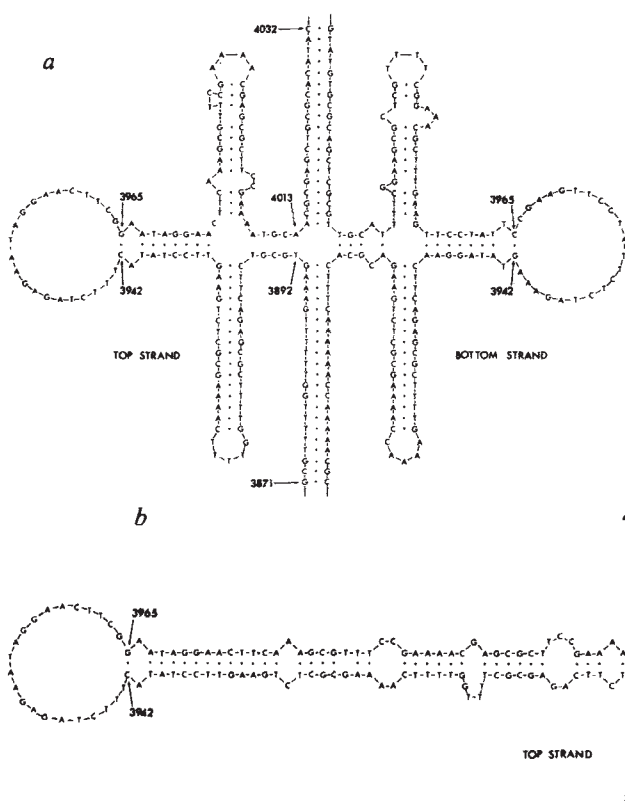
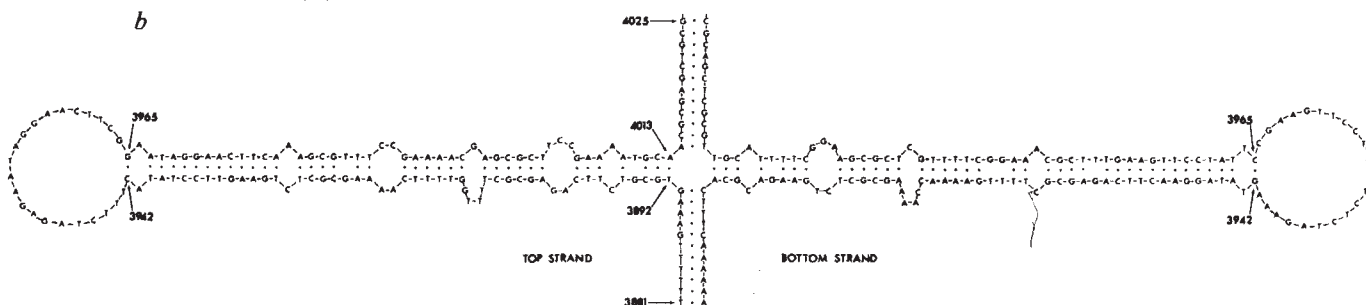


Fig. 3 a, b, Alternate potential secondary structures of the DNA sequence near the putative origin of replication. Both structures would have theoretical thermodynamic stabilities of the order of $-50 \text{ kcal mol}^{-1}$ (ref. 33).



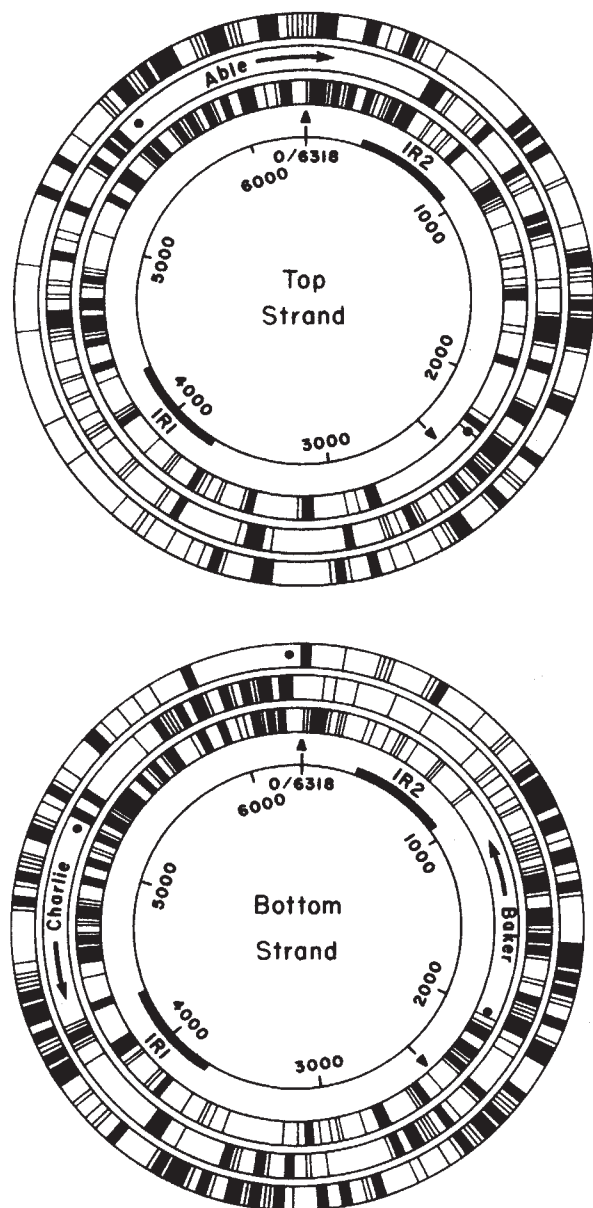


Fig. 4 Approximate positions of stop codons in the different reading frames of the yeast plasmid. TAA codons are represented by blocks (■), while TAG and TGA codons are shown as lines. Start codon positions for 'Able' and 'Baker', and 'Charlie' and two other large open reading frames are indicated by dots (●). The outer, middle and inner rings correspond to reading frames 1, 2 and 3, respectively. For the top strand reading frames 1, 2 and 3 begin with the codons GAA (positions 1-3), AAT (2-4) and ATT (3-5), while for the bottom strand reading frames 1, 2 and 3 begin with codons CTC (6,318-6,316), TCA (6,317-6,315) and CAA (6,316-6,314). Triangles indicate *EcoRI* cleavage sites.

a unique opportunity to study eukaryotic recombination at the molecular level.

A second observation of Gerbaud *et al.*²² has implications for the construction of yeast transformation vectors. They determined the levels of expression of a yeast gene (*ura3*) inserted at two different points in their vector. It was found that the *ura3* activity per transformed cell was three times higher when the gene was inserted at the *EcoRI* site within coding region 'Able' than when it was carried within the bacterial plasmid segment of the vector. Since the 'Able' product is apparently actively expressed in yeast (above) it is reasonable to suppose that the higher expression was due to efficient transcription utilizing the 'Able' promoter or other transcriptional signals.

In view of these considerations, some attributes of a vector-host system for the transformation of yeast may be suggested. The host should be [*cir*⁺] since high transformation frequency and reasonable stability are obtained^{19,22}. The vector should contain the origin of replication but should not contain the entire yeast plasmid, since Toh-e *et al.* have shown that increased stability results from this arrangement when the host is [*cir*⁺] (ref. 25). The vector should also contain coding region 'Able' for the insertion of foreign DNA to be expressed in the yeast host. The sequence indicates that the small *AvaI* fragment of the form B plasmid would have all these properties. This 2,945 base pair fragment would contain the origin of replication and the entire 'Able' coding region, as well as segments flanking both ends of 'Able'. Within the 'Able' sector there are single sites for the restriction enzymes *EcoRI*, *HindIII*, *BalI*, *BclI* and *ClaI* which might be suitable for expression of foreign genes. In combination with a suitable selection marker(s) the resultant plasmid should possess improved properties for cloning foreign DNAs in yeast.

The sequence of the yeast plasmid may improve the usefulness of yeast for DNA cloning experiments. In addition, the sequence provides a starting point for further investigations of such functions as replication, recombination and gene control in a well characterized eukaryotic genetic system.

Copies of the sequence with a complete listing of all restriction sites are available on request.

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Addendum from J. Hindley, T. C. Elleman & G. A. Phear

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The accompanying article by Hartley and Donelson presents the sequence of the type A yeast plasmid recovered from *Saccharomyces cerevisiae* strain A364A D5 by cloning into pMB9. We have independently determined about 63% of the type A plasmid sequence from pJDB 71 (ref. 1; a hybrid constructed by inserting the 2μ plasmid from *S. cerevisiae* strain DR19/8T into the *EcoRI* site of pMB9), using a different approach and have compared the two sets of data. This is of interest for two reasons. First, Hartley and Donelson used the Maxam–Gilbert procedure whereas we have relied entirely on the dideoxynucleotide chain termination procedure using single-stranded templates generated by cloning restriction fragments into linearized phage M13mp2 RF-DNA and subsequently isolating the single-stranded recombinant phages². The sequence comparison was made over a total of 4,004 nucleotides comprising sequences totalling 3,053 nucleotides from the larger and 951 nucleotides from the smaller unique regions of the plasmid. Both sets of data were in complete agreement. This result therefore completely validates

the reliability of the newer sequencing procedures used in this work. Neither during propagation of the hybrid plasmid nor in the subsequent M13 cloning and primed syntheses is there any evidence of rearrangements or deletions of sequences.

Second, the data imply that the unique regions in the plasmid, bounded by the inverted repeats, are fully conserved in two unrelated strains of yeast. This identity includes a sequence of 863 nucleotides extending from the *EcoRI* site at position 2,407 to position 3,270. This region contains a set of 6.5 direct tandem repeats of 62–63 residues and in two other plasmid isolates (Scp 2 and Scp 3)³, which contain deletions of about 130 and 220 base pairs respectively, the mapping data puts both these deletions within the region of tandemly repeated sequences. The available data therefore suggests that, apart from this deletable region, the unique regions of the plasmid are strongly conserved. Differences are found however when the inverted repeat regions are compared. All these seem to be single base changes and occur in regions outside the possible reading frames suggested by Hartley and Donelson (see ref. 4).

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LETTERS

The double quasar 0957 + 561 as a gravitational lens: further VLA observations

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The twin quasars 0957 + 561 A,B, separated by only 6 arc s on the sky, have been shown to have nearly identical redshifts ($z_e \approx 1.41$, $z_s \approx 1.39$) by Walsh *et al.*¹, who suggested that they were the double image of a single object, split by the gravitational refraction of an unseen massive intervening object. Further optical spectroscopy² has confirmed the remarkable similarities of the emission and absorption spectra of the two quasars, and strengthened the case for a gravitational lens model. Radio maps at 6 cm made with the Cambridge 5-km telescope³ and with the VLA⁴ showed that, in addition to two unresolved sources coincident with the optical quasars, there were extended regions of radio emission, apparently associated with the north (A) quasar which greatly complicated the gravitational lens hypothesis. We now report a full 12-h synthesis made at the VLA at 6-cm wavelength⁵ which confirmed the earlier maps and showed additional features at lower flux levels. In particular, this map revealed two small radio 'jets', one close to each of the quasars. The absence of corresponding second images of either of these jets near the other quasar was used to rule out models in which the gravitator was located near the midpoint of the line between A and B (ref. 5).

The lens hypothesis was boosted by the discovery^{6,7} that the field of 0957 + 561 was overlaid by a rich cluster of galaxies at $z \approx 0.39$. Furthermore, the nucleus of the brightest member of the cluster (designated G1) lay ~ 1 arc s north of the B quasar, very near the position of the B radio jet. Young *et al.*⁷ presented models for gravitational imaging of the underlying object quasar, invoking both the properties of an extended gravitator

(G1) and the existence of an additional source of bending (the cluster as a whole) and were able to account for most of the observed features. The B jet, however, remained difficult to understand as part of the gravitational image of the quasar and its extended radio emission. The present results clarify the nature of the B radio jet, and further strengthen the gravitational lens interpretation of the double quasar.

The observations consist of a full 12-h synthesis made at 6 cm on the Very Large Array of the National Radio Astronomy Observatory during 22–23 February 1980. A total of 22 antennas were functioning, with baselines ranging between 45 m and 22.4 km. Observing conditions were ideal, and the data were of significantly higher quality than those obtained in October 1979⁵. The performance of the array was monitored by frequent observations of the nearby point source 1031 + 567 and the flux scale was set by assuming the 4,885 MHz flux density of 3C286 to be 7.41 Jy. Editing, gridding, and Fourier transformation of the data were carried out using the standard VLA data reduction procedures. The resulting 'dirty' map was processed with the modified CLEAN algorithm of Clark⁸. The clean map, shown in Fig. 1, has a resolution of 0.66 by 0.41 arc s FWHM, and a dynamic range (map peak/r.m.s. noise) of $\sim 200:1$.

The significant feature of Fig. 1 is the resolution of the B jet into a point source (which we now denote G) of 2.3 ± 0.2 mJy flux, located 1.06 ± 0.02 arc s N and 0.15 ± 0.02 arc s E of the B quasar. The position and flux of G are consistent with our previous observations⁵. An optical photograph of the field, made on the University of Hawaii 2.2-m telescope by Stockton⁹, puts the optical nucleus of G1 0.99 ± 0.03 arc s N and 0.19 ± 0.03 arc s E of the B quasar, or 0.08 ± 0.05 arc s SE of the radio position. Thus, the optical nucleus of G1 and the radio source G are coincident within the small uncertainties. The simplest interpretation is that G is a compact radio source in the nucleus of G1 and is not part of the gravitational image. If this is so, the 5 GHz power of G is $P_{5000} = 1.1 \times 10^{-23}$ WHz⁻¹ sr⁻¹ ($q_0 = 1/2$, $H_0 = 50$ km s⁻¹ Mpc⁻¹, spectral index assumed to be 0.25). Although little is known about the compact radio structure of cD galaxies selected without regard for their low-frequency properties, G is comparable to the central components of 3CR radio galaxies¹⁰. Although there is at least one other interpretation (see below), the fact that G is not significantly north of the nucleus of G1 removes the last strong objection to the gravitational lens explanation of 0957 + 561.

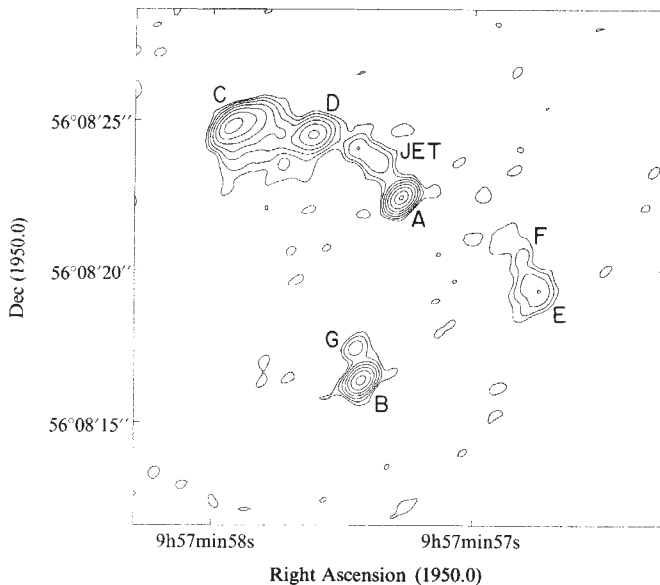


Fig. 1 The double quasar 0957+561 A,B, mapped at $\lambda 6$ cm by the VLA on 22–23 February 1980. The point sources A and B are coincident with the optical quasars, and the small source G lies very close to the nucleus of the intervening galaxy G1 which is apparently forming the double image. The contour levels are 1, 2, 4, 8, 16, 32, 64 and 95% of the peak flux of 35.9 mJy.

We now consider how well the details of the gravitational imaging are understood, what further data may be needed, and what more can be learned from the double quasar. The important features to be accounted for are: (1) the near-equality of the fluxes of A and B despite their very different separations from G1; (2) the absence of second images of any of the extended radio emission; (3) the lack of resolution of the B image by the VLA or by existing VLBI observations; and (4) the fact that the nucleus of G1 lies slightly (0.4 arc s) off the line between A and B. Young *et al.*⁷ have suggested that the first two points are consequences of the existence of a caustic for an extended gravitational lens. In their picture, the B image is formed very close to the caustic and is, therefore, anomalously amplified, while the extended emission lies beyond the caustic so no second images are formed. This model has the additional consequence that the B image must itself be multiple. The VLBI observations of Porcas *et al.*¹¹ and Haschick *et al.*¹² show the existence of compact components in both A and B, but have insufficient dynamic range to give useful details of their brightness distributions. The high dynamic range of the observations presented here enable us to constrain a possible double nature of B. There is no evidence for a broadening of B relative to A at a level of 5% of the beamwidth. Thus, if two images B and B' are of equal intensity ($I = I'$), their separation $\delta\theta$ is less than 0.16 arc s. We are, therefore, unable to rule out model 'c' of Young *et al.* in which $I/I' = 1.45$ and $\delta\theta = 0.15$ arc s.

Young *et al.* have emphasized that the cluster as a whole may play an important role in forming the image, and they attribute property (4) above to the effect of the cluster. As a result of the cluster, one of their models results in the observed B image plus a very faint B' image nearly coincident with G1. This may be the correct interpretation of the small radio source G. If in fact G is north of the nucleus of G1, G cannot be the third image B'. In addition, note that the absence of second images of the extended emission must be used to constrain the role of the cluster, and Young *et al.* did not consider the radio data in constructing their models.

Improved VLBI experiments currently underway will constrain models in which B is double with separation less than the 0.16 arc s detectable in the current VLA data. In addition, measurement of the time delay between the arrival of the A and B images will help ascertain the importance of the cluster in the image formation. The differential delay, expected to be of the

order of months in the absence of the cluster, could be multiplied by a factor of the order of 5 by the effect of the cluster⁷. In principle, this sensitive probe of the cluster could yield information on any 'hidden' mass in the cluster⁵. The intensity ratio $I_A/I_B = 1.23 \pm 0.02$ in the data presented here, while it was 1.32 ± 0.02 in October 1979 (ref. 5), so there seems to be measurable variability in the underlying quasar, making a time-delay measurement feasible. Finally, we note that cD galaxies such as G1 can have double or multiple nuclei, and hence may have appreciable non-radial refraction effects. Thus it may not be necessary to invoke strong bending due to the cluster in order to understand the slight misalignment of A–G1–B.

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Optical spectroscopy of interplanetary dust collected in the Earth's stratosphere

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A new class of extraterrestrial material has recently become available for study: interplanetary dust particles (2–30 μ m in size) collected in the atmosphere at 20-km altitude by inertial impactors mounted on NASA U-2 aircraft in the programme of sampling initiated by Brownlee *et al.*¹. The extraterrestrial nature of a compositionally 'chondritic' subset of these particles has been verified by measurements of the elemental and isotopic abundances of noble gases^{2,3}. We report here the first optical absorption spectra of these interplanetary dust particles (IDPs). We show that absorption features in the visible at $\sim 9,800$ cm^{-1} and in the IR at $\sim 1,000$ cm^{-1} are present in spectra of these particles. An $\sim 1,000$ cm^{-1} feature is present in optical spectra of meteorites, comet dust and interstellar dust, although its cause in the latter two is a subject of some debate^{4,5}. Future experimental work on these laboratory samples promises to shed light on the alternatives, at least for particles in the Solar System dust cloud.

Particle handling techniques are described elsewhere⁶. After removal from collectors supplied by Brownlee, particles were individually cleaned of the silicone oil collecting medium by immersion in a running stream of hexanes. They were mounted and characterized briefly by optical microscopy, scanning electron microscopy and energy dispersive X-ray analysis before

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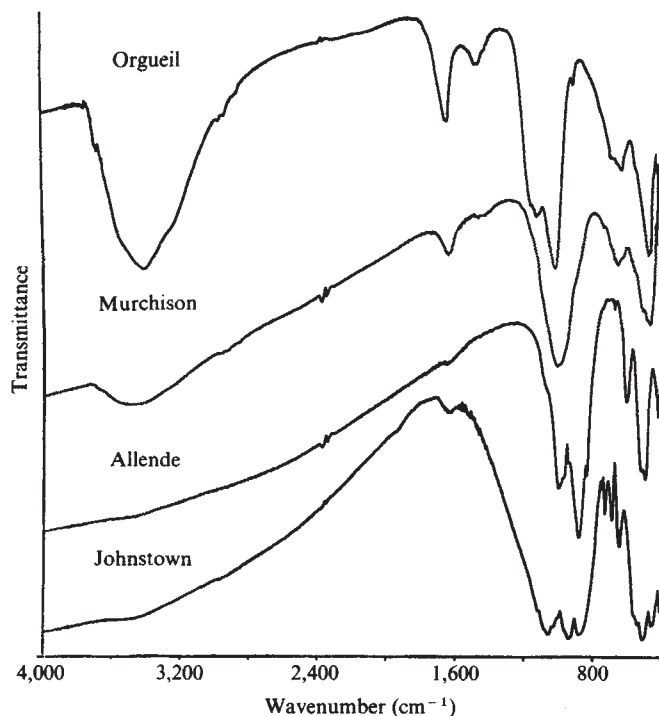


Fig. 1 IR transmission spectra of different meteorites at room temperature. The samples were prepared by mechanically grinding a 1-mg sample with 100 mg of KBr and forming a clear pressed pellet by the standard IR-KBr pellet preparation technique. A commercial Nicolet 7199 FTIR spectrometer equipped with a globar source and mercury-cadmium-telluride detector was used to record the IR spectra.

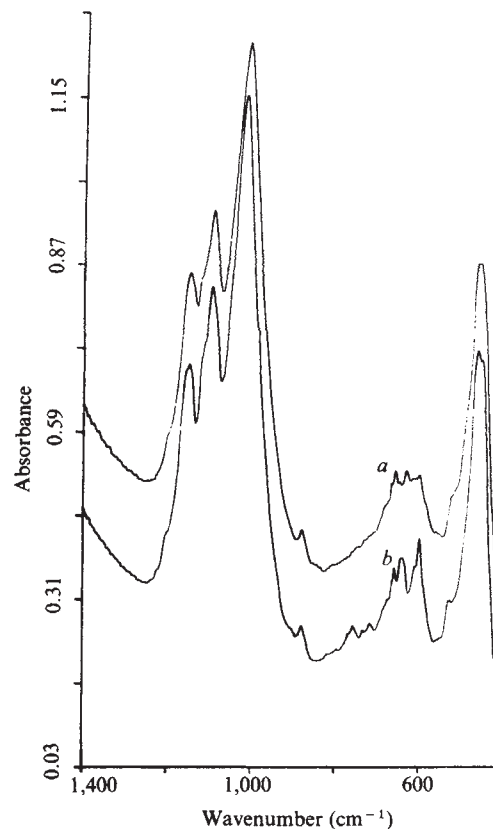


Fig. 2 IR transmission spectra of the Orgueil meteorite: *a*, at room temperature (25°C); *b*, at -168°C.

being crushed and mounted for the measurement of optical properties using Fourier transform spectroscopic methods.

Absorption spectra of meteoritic materials in the mid-IR show a wealth of detail. Figure 1 shows room temperature (25°C) IR spectra from several different meteorites between 4,000 and 400 cm^{-1} obtained using large (10^{-3} g) samples. Similar spectra have been published previously⁴. The structured absorption feature centred near $\sim 1,000 \text{ cm}^{-1}$ is probably due to Si-O stretch bands of meteoritic silicates. The spectra of these samples were then recorded at near liquid nitrogen temperature (-196°C) to simulate the lower temperatures found in space. Figure 2 compares the low temperature (-168°C) and room temperature spectra of the Orgueil meteorite. At low temperature, the weaker bands become sharper and better resolved, but the distinguishing spectral features are not significantly altered.

An IR spectrum of pieces from three IDPs (total sample $\sim 10^{-9}$ g) is shown in Fig. 3*a*. A broad feature in the region 1,300–800 cm^{-1} is clearly present. Figure 3*b* shows a comparison spectrum of small particles from the Orgueil meteorite taken under similar circumstances. Possible causes for the IDP feature include mafic silicates⁴ and carbonaceous material⁵ which are known to be present in these particles^{7–9}.

Figure 4 shows the visible/near IR spectra of several carbonaceous meteorites, and of a sample composed of five crushed IDPs (total sample $\sim 3 \times 10^{-9}$ g). The well studied feature centred at $\sim 9,800 \text{ cm}^{-1}$ in the meteoritic spectra is due to crystal field transitions of iron¹⁰. Comparisons of the IDP and the carbonaceous chondrite spectra show that the absorption spectra are different, in spite of the similarities in elemental abundances of the two classes of object⁷. A weak iron crystal field absorption band is present in the IDP spectrum.

A prominent $\sim 1,000 \text{ cm}^{-1}$ feature is a commonly observed feature of dust found in different astrophysical settings⁴. Although it may never be possible to establish definitively the nature of the material (or materials) responsible for such an absorption in interstellar dust, it seems likely that the problem

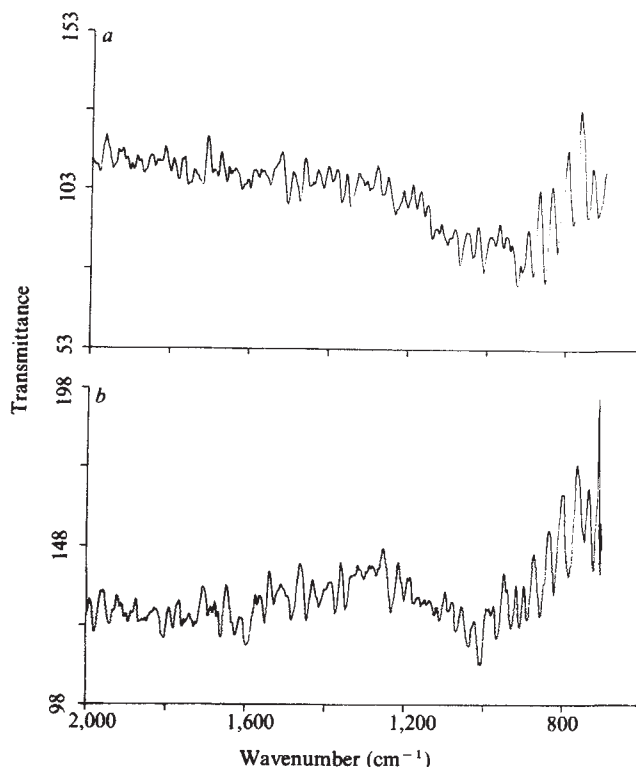


Fig. 3 IR transmission spectra of: *a*, IDPs; *b*, a particle from the matrix of Orgueil. Spectra were acquired by crushing the particles onto a thin ($\sim 100 \mu\text{m}$) freshly cleaved KBr plate using a glass slide and centering them in a $50 \mu\text{m}$ aperture¹¹. The sample mount was then placed in a $4\times$ beam condenser using an $f/1$ KBr lens and aligned by adjusting the sample position until the detector signal was maximized. Spectra were obtained by 20,000 time averaged scans with 8 cm^{-1} resolution.

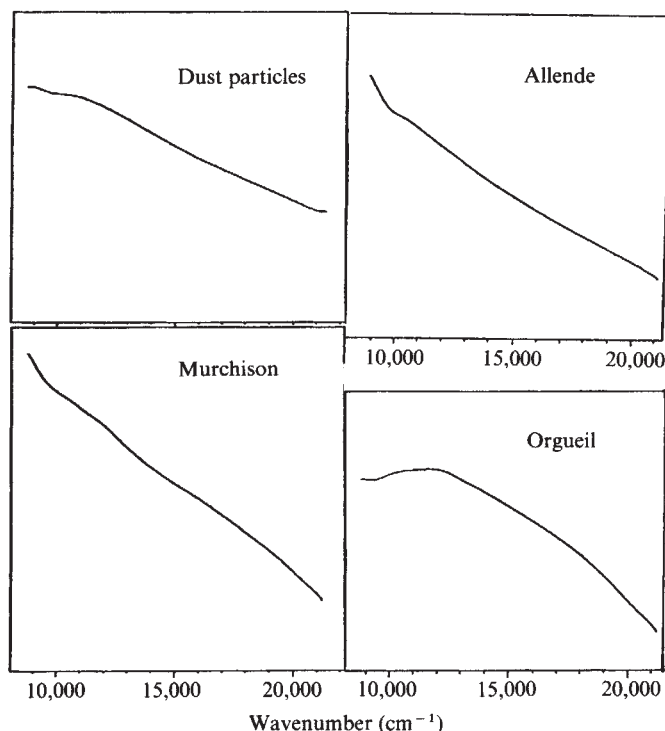


Fig. 4 Visible/near-IR spectra of powdered samples from IDPs and several carbonaceous chondrites. Ordinate values are proportional to the ratio of light transmitted through the sample and light transmitted through a blank. The spectra were obtained using the solar FTS at the Kitt Peak National Observatory. The instrument is a dual beam FTS having input and output focal lengths of 2.9 and 2.3 m, respectively. The beam splitter was silver on quartz and an entrance aperture ($f/50$) of 4 mm was used. Transmission measurements were made on powdered samples which were immersed in silicone oil and sandwiched between glass slides, separated by spacers. Illumination was provided by a 150 W tungsten-halogen lamp.

can be solved for interplanetary dust. Although the collected IDPs have certainly experienced some thermal alteration during deceleration in the Earth's atmosphere, the particles seem to retain absorption features found in cosmic dust. Work is in progress to separate, or at least remove selectively, the various constituents of the IDPs to evaluate separately their contribution to the absorption. A severe constraint is posed by the small size of the particles and their present rarity. The particles are also known to differ considerably in their mineral assemblages from one particle to the next⁹. Fourier transform spectroscopy on individual particles may prove to be an important future technique for the nondestructive classification of particles according to type.

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Solar variability and climatic change during the current millennium

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Claims of a Sun-weather relationship¹ can only be evaluated properly when the history of solar change is known in detail. The most obvious feature of solar variability is the change over time in the number of sunspots on the visible half of the Sun. A less visible indicator of solar change is the ¹⁴C content of tree rings. The atmospheric ¹⁴C levels derived from tree ring measurements can be tied to the Sun's modulation of the cosmic ray flux in the vicinity of the Earth, and thus provide a history of solar change.² We report here a comparison of climate records with the record of solar change obtained from atmospheric ¹⁴C variations. This yielded 'negative' results, that is, a relationship between the climatic time series and the ¹⁴C derived record of solar change could not be confirmed.

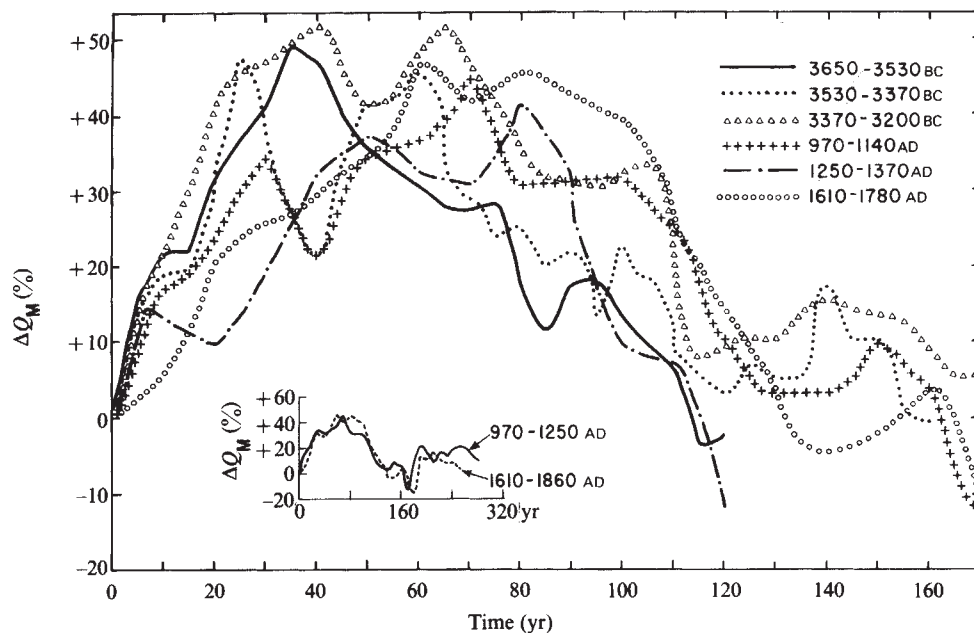
The modulation of the cosmic ray flux (and the associated atmospheric ¹⁴C production rate Q in atoms $s^{-1} cm^{-2}$ of the Earth's surface) by the Sun occurs through changes in the magnetic shielding properties of the solar wind. However, this is not the only cause of atmospheric ¹⁴C change. The Earth's geomagnetic field also influences the pathways of the charged particles that constitute cosmic radiation. Both solar modulation and longer-term geomagnetic field changes have to be taken into account when decoding the atmospheric ¹⁴C signal. A carbon reservoir model^{2,3} that describes terrestrial carbon exchange between the atmosphere, ocean and biosphere can be used to calculate from the atmospheric record the production rate changes ΔQ_M . In carbon reservoir modelling, the long-term trend in the atmospheric ¹⁴C attributed to Earth geomagnetic dipole changes is removed by deducting the sinusoidal approximation given by Houtermans⁴. The remaining ¹⁴C variations are used to calculate the ¹⁴C production rate changes ΔQ_M around the steady state value of 1.57 atoms $cm^{-2} s^{-1}$ from the model. The magnitude of the ΔQ_M changes caused by solar modulation depends on the Earth's geomagnetic field intensity because the average global ¹⁴C production becomes less sensitive to solar modulation when the Earth's geomagnetic field increases^{5,6}. The ΔQ_M values given here are normalized to the present-day Earth's geomagnetic field intensity by using the correction factor $[M(0)/M(t)]^{0.63}$ derived from data given by O'Brien. The magnetic field changes were obtained from data given by Barton *et al.*⁷.

Climate-related changes in carbon reservoir exchange rates may also cause atmospheric ¹⁴C levels to change. The calculated record of ¹⁴C production rate changes (ΔQ_M) may, therefore, contain 'fictitious' rate changes that are not related to solar modulation, but to climate changes. The contribution to the ΔQ_M record of such fictitious ΔQ_M changes is difficult to evaluate, but it can be argued that climate variability is not a dominant factor in producing century-type atmospheric ¹⁴C variations^{2,8}.

The changes in calculated ¹⁴C production rates generally agree with the evidence for solar change obtained from historical observations of sunspot number and aurorae. For instance, there are three intervals in the post AD 1200 sunspot record where visual sunspot observations are lacking (the Wolf, Spörer and Maunder minima). For each interval of reported low incidence of sunspots, the ΔQ_M record reaches a maximum. There is also covariance between ¹⁴C production rate changes and sunspot numbers for the eighteenth and nineteenth centuries².

When discussing the ¹⁴C changes, note that the standard deviation in the ¹⁴C measurements is ~1.5-2%. This corresponds to an average standard deviation in the calculated

Fig. 1 ^{14}C -production rate changes ΔQ_M of solar oscillatory modes that are similar to the Maunder minimum mode. The ΔQ_M values are the increases over the preceding lowest ^{14}C production rate. The ^{14}C production rate changes were normalized on the present-day Earth geomagnetic field intensity (see text).



ΔQ_M values of $\sim 4\%$. It is possible to recognize many similarities within the stated precision in the ^{14}C pattern. The ^{14}C production rate changes are, therefore, not random fluctuations.

Two of the ΔQ_M oscillations in the current millennium are, within the experimental error, copies of each other. These are the variations encountered between AD 970 and 1250, and between AD 1610 and the end of the nineteenth century (see Fig. 1). The similarity of the Fig. 1 curves implies that the 'grand medieval maximum' of the twelfth century⁹ has the same level of solar activity as the AD 1740–1840 interval. The Sun switches near AD 1250 to the quiet Wolf minimum, whereas the Sun is still active after AD 1890. The agreement between the curves terminates, therefore, after 280 yr.

The close correspondence of some of the ^{14}C record puts limitations on the theories of solar dynamo behaviour. Dynamo theories may include the possibility that the envelope of the solar cycle, and the associated magnetic field in interplanetary space, is inherently unpredictable¹⁰. The repetitive pattern seen in the ^{14}C record argues against this interpretation.

Many of the oscillations are similar to the late seventeenth century Maunder minimum pattern. The ΔQ_M oscillations between AD 970 and 1100, AD 1250 and 1370, and AD 1610 and 1750 all have the same magnitude, approximate duration and shape (Fig. 1). The geomagnetically normalized ΔQ_M variations, calculated from a ^{14}C record¹¹ between 3650 and 3300 BC are also given in Fig. 1. The 3650–3520 BC, 3530–3370 BC, and the 3370–3260 BC oscillations again have similar magnitudes and duration. The times necessary to reach the maximum production rate change are different. This rise-time is 40 yr for the BC oscillations and 50–70 yr for the more recent ones.

Three of the four major oscillations of the current millennium are of the Maunder minimum type, lasting 120–140 yr. The remaining oscillation is the Spörer minimum, lasting nearly twice as long (240 yr). The Spörer oscillatory mode is seen less frequently than the Maunder mode. The ^{14}C record given by Suess¹² suggests that the preceding Spörer mode occurred as long ago as 700 BC.

A spectral analysis of the post-AD 730 record yields the curve in Fig. 2a. The probability of exceeding, in a random distribution, the peak sizes with periods of about 130 and 200 yr are, respectively, 1.4 and 4.9 %.

The so-called 'Little Ice Age' in Europe in the seventeenth century is sometimes attributed to the solar changes that took place during the Maunder minimum⁹. If indeed the Sun does influence climate in this manner, then there should be many more Little Ice Ages because the solar Maunder mode is quite

prevalent. One would then also like to postulate a cooling of double duration for the less frequent double Maunder (Spörer) mode. In fact, with four major oscillations during a millennium, it would be conceivable that 40-odd little ice ages existed during the current Interglacial. Furthermore, one would expect similarities in climatic response for certain periods, such as the AD 970–1250, and the AD 1610–1890 intervals.

Figure 3 shows the solar-related ΔQ_M record compared with eight climate records. Two of these are based on oxygen-isotope ratio measurements in ice cores. The derivation of the ages of

Table 1 Correlation coefficients r derived from cross-correlating the ^{14}C production rate record given in Fig. 3 (ΔQ_M curve) with the climate records listed below

Fig. 2	Locality	r	$r_{\max}$	Δt (yr)	$r_{\max}$	Δt (yr)
a	Oxygen isotopes ice core, Devon island ¹⁴	+0.08			+0.25 (5%)	50
b	Oxygen isotopes ice core, Camp Century, Greenland ¹⁵	-0.19	-0.43 (3.5%)	-30	+0.33 (19%)	90
c	Oxygen isotopes ice core, Crete, Greenland ¹⁶	-0.01			-0.26 (5%)	50
d	Tree ring width, Lapland ¹⁷	-0.01	-0.33 (6%)	-90	+0.36 (3%)	50
e	Winter severity index, Russia ¹⁸	-0.14	-0.43 (1.6%)	-30		
f	Tree ring density, Lauenen, Switzerland ¹⁹	+0.05	-0.31 (10%)	-90		
g	Winter severity index, UK ¹⁸	-0.03				
h	Mean annual temperature, UK ¹⁸	-0.06				
i	Deuterium isotopes tree rings, White Mountains, Nevada ²⁰	-0.11				
j	Tree ring width, White Mountains, Nevada ²¹	-0.32	-0.32 (5%)	0	-0.32 (5%)	0

The $r_{\max}$ values are obtained by assuming (1) climate lags the ΔQ_M curve (Δt positive) or (2) climate-influencing variables precede the Q changes (Δt negative). The probabilities of exceeding the listed r values in a random series are given in parentheses. $r_{\max}$ values are only listed for time lags of 100 yr or less.

the Camp Century core (Fig. 3b) and Devon Island (Fig. 3a) are mainly based on ice flow dynamics. The time scale of all other curves is based on either tree ring counting or historic dating. Ages determined in this manner should be considered more accurate than those assigned to the oxygen isotope curves.

The climate curves in Fig. 3 were constructed from available records that are listed in Table 1. These records give information on regional climate only. They are used for a comparison with the solar record because other, more representative, indicators of global climate are not available over such a long interval.

A downward move of the climatic parameters in Fig. 3 implies in all instances a colder environment. The solar ΔQ_M record is plotted on an inverse scale, mainly to facilitate a visual investigation of a possible relationship between reduced solar activity (higher ^{14}C production rates) and colder climate.

Conclusions that can be drawn from the comparisons made in Fig. 3 are:

(1) The detailed work of Schweingruber *et al.* on tree ring density¹⁹ does not agree with contemporaneity of the Maunder minimum and the climatic cooling of the seventeenth century that is often referred to as the Little Ice Age. The tree-ring density record points to an extended cold episode between AD 1570 and 1640. This interval coincides with the period of high solar activity (low ΔQ_M values) between the Spörer and the Maunder minimum. Several of the other climate records given in Fig. 3 also have a tendency towards colder weather during this interval of high solar activity.

(2) During the Sun's switch to the less active Spörer and Maunder minimum modes, Russia especially experienced colder winters.

Correlation coefficients derived from cross-correlating the climate and solar curves are small in nearly all cases. These coefficients r are given in Table 1, which also lists the oxygen isotope record of Crete, Greenland¹⁶ and a Lapland tree-ring width series¹⁷, not shown in Fig. 3, for which the r values are both -0.01 . The White Mountains' ring width data cross-correlate with the solar record at the 5% significance level. The other correlation coefficients indicate a complete absence of a direct relationship between the solar Q and climate curves.

By assuming a time lag between climate and the solar curves, it is possible to obtain larger correlation coefficients for several of the time series listed in Table 1. There are two possibilities: either climate lags the solar curve (Δt positive) or the solar properties that possibly influence climate precede the Q changes (Δt negative). Table 1 lists those Δt values ($|\Delta t| \leq 100$ yr) for which r reaches a maximum value r_{max} .

Because the Q record has substantial autocorrelation (it takes a 50-yr lag for the autocorrelation coefficient to approach zero) the normal test of significance is no longer applicable. Autocorrelation reduces the number of independent observations used in interpreting the derived cross-correlation coefficients. The effective number N of independent observations were calculated for each time lag according to the procedures discussed by Quenouille²². The number N was subsequently used in deriving the probabilities of exceeding the Table 1 r_{max} values.

Table 1 lists nine climate series of different regions (series h is partially derived from series g and has not been included in the following discussion). One hundred and eighty-nine comparisons were made by lagging the data up to 100 yr (21 comparisons with the Q record for each climate series). In a

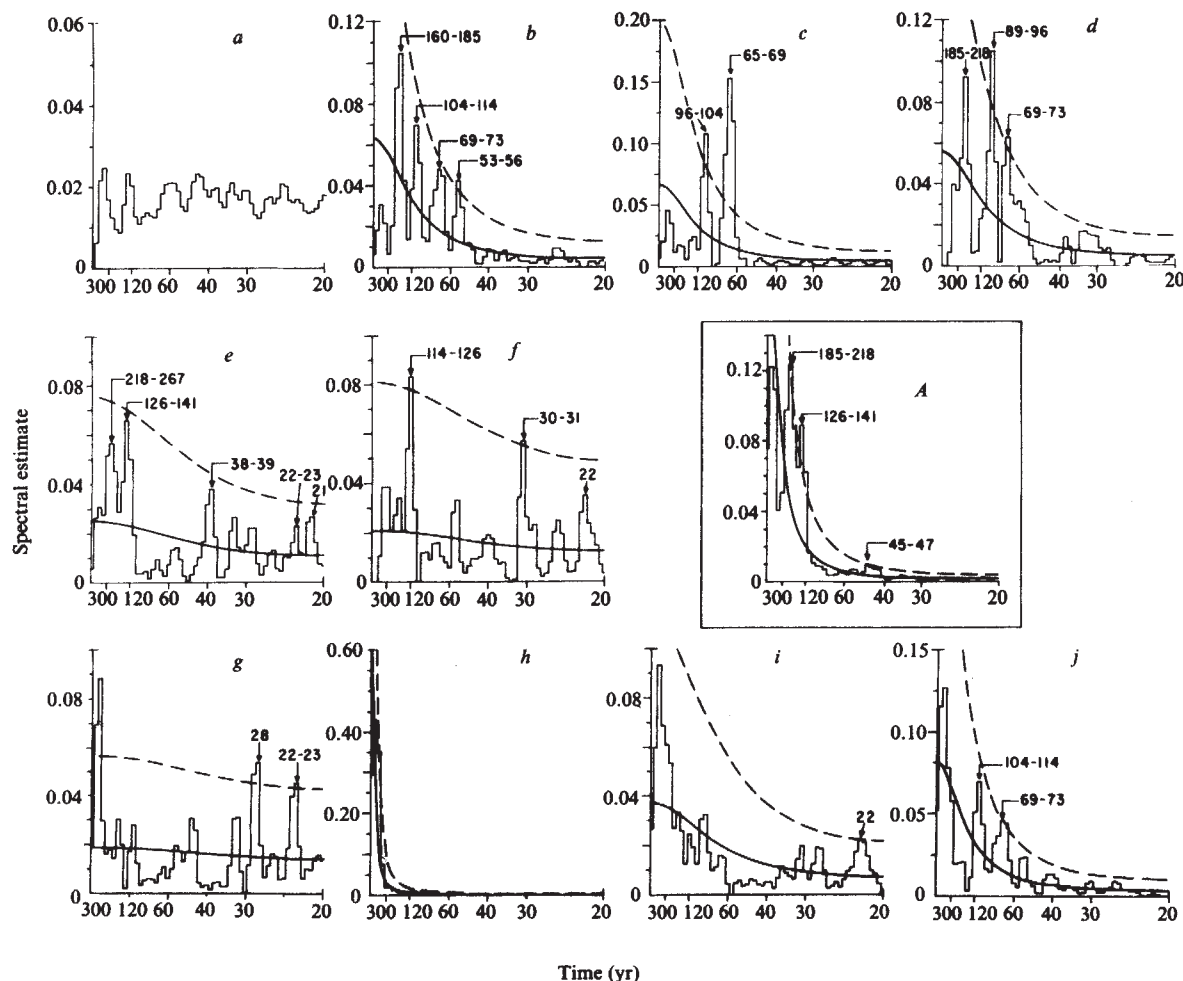


Fig. 2 Spectral distribution of the post-700 AD ^{14}C production rate record (curve A) and climate records (curves a-j). The various climate records are described in Table 1. The dashed line gives the 2σ confidence limit whereas the solid line gives the red noise continuum¹³.

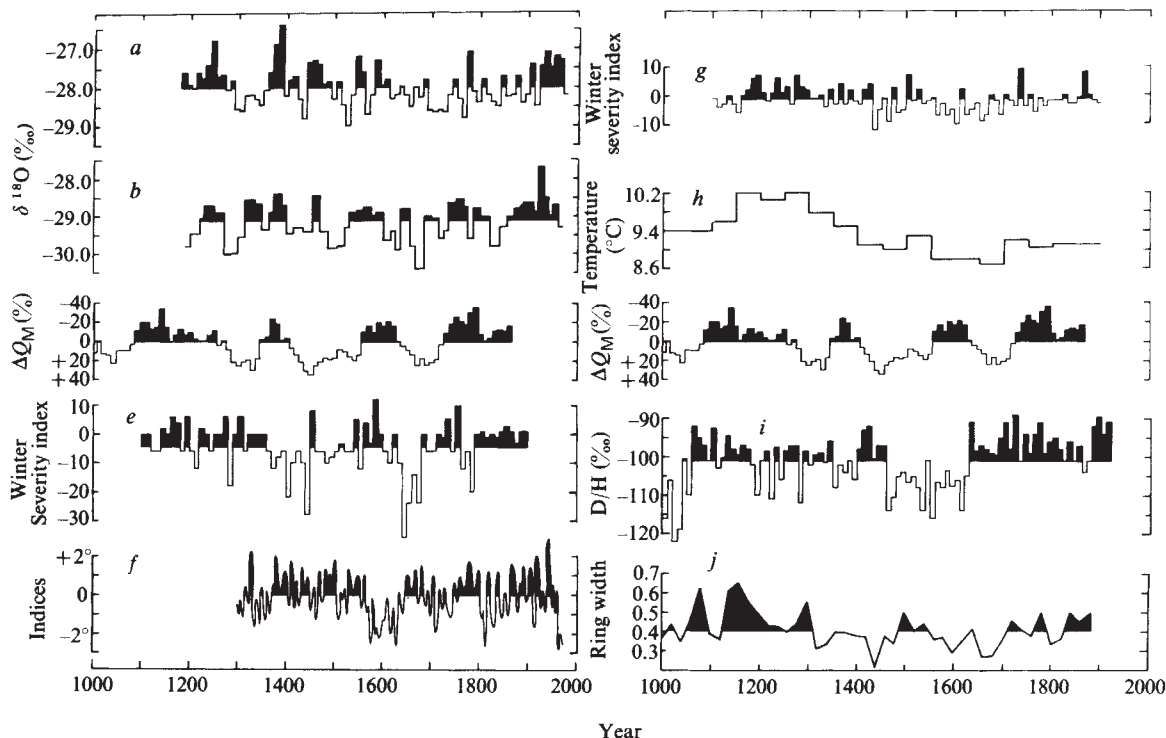


Fig. 3 A comparison of climate and ^{14}C production rate records. The various climate records are described in Table 1.

comparison of non-related time series, the distribution of correlation coefficients should be 1, 9, 51 and 128 for, respectively, the 0–0.3%, 0.3–5%, 5–32% and 32–100% probability intervals. The Q and climate comparisons yield a distribution of 0, 12, 50 and 127 correlation coefficients for the intervals listed above. The combined lagged climate records therefore do not cross-correlate with the Q record beyond random statistics.

Another approach to the investigation of a Sun–weather relationship is to look for common periodicities in the solar related ΔQ_M record and the climate records. The climate records contain several periodicities (Fig. 2). The closest agreement in spectral distribution between the solar related ^{14}C (curve A) and climate records is obtained for the Russian winter severity index (curve e). The spectra of other high-latitude regions, such as Camp Century, Greenland (curve b) and Lapland (curve d) also partially agree. On the other hand, the climate spectra also have several periodicities that are not present in the ^{14}C derived solar record. One of these periods, near 70 yr, is found in four climate records. Note that the 22-yr cycle, already discussed by Epstein and Yapp for the D/H record (curve i) is also present in the winter severity indices of England and Russia (curves g and e), and the Swiss tree-ring record (curve f). The 22-yr cycle is absent in the ^{14}C production record (curve A) but this is due to a lack of sensitivity for the faster oscillations (a rapid decadal production rate change is attenuated more, and results in atmospheric ^{14}C variations of a few per mil only).

Earlier studies of the ^{14}C and climate record^{9,23–25} suggested a direct relationship between the solar modulated atmospheric ^{14}C levels and climate. These studies did not take into account the variable time lag between the atmospheric ^{14}C levels and the ^{14}C production rate changes. The precision of the ^{14}C record used in those investigations also did not allow for a detailed study of a phase shift between solar input function and climate. The high precision ^{14}C measurements reported here do not support a statistically significant relationship between the combined regional climate and ^{14}C time series.

Many factors influence local climate change, and a solar influence on climate can perhaps be expected in a few areas only. The Camp Century and Russian climate records demonstrate on the basis of the statistical analysis a climate solar relationship

(albeit 30 yr out of phase). A selective comparison of solar and climate records, however, can only be justified if a mechanism can be found that explains the postulated increased sensitivity to solar changes of these areas. Perhaps jet stream oscillations that seem to relate to the 11-yr solar cycle²⁷ may provide some answers in this direction.

The length of the records discussed above does not allow for the investigation of a solar–climate relationship with periodicities of the order of millennia²⁶. Sun–weather effects may still also be tied to other parameters of solar change, such as the umbra–penumbra ratios discussed by Hoyt²⁸.

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Modern climate–tree-growth relationships and climatic reconstruction in sub-Arctic Alaska

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Statistical comparisons between tree-ring width sequences and climatic records provide a means of identifying climatic limitations on tree growth and allow the reconstruction of past climates. This information is especially important in the North American sub-Arctic where climate–growth relationships are poorly understood and instrumental weather records are very short, typically less than 75 yr. Dendroclimatic reconstructions before 1900 are essential for estimating a realistic range of high latitude climatic variation, because twentieth century climate is now thought to be somewhat anomalous¹. While some dendroclimatic studies have been carried out in the sub-Arctic^{2–8}, none has made full use of current multivariate statistical techniques. This study, as part of a multidisciplinary investigation of treeline fluctuations in the Brooks Range of Alaska, uses ring-width sequences of white spruce (*Picea glauca* [Moench] Voss) to define climatic limitations on radial growth at treeline and to reconstruct past climatic variables for Fairbanks, Alaska. Multiple regression techniques were used to analyse climate–growth relationships and the results suggest important modifications to the theory that growth at treeline is limited primarily by summer temperature^{2,8,9}. For example, we have found that radial growth is directly related to summer and autumn temperature and precipitation during certain months, but is inversely related to winter–spring temperature. The same ring-width sequences were used to reconstruct average May–July temperature at Fairbanks, Alaska, for the period 1829–1930. This reconstruction, which more than doubles the length of the existing climatic record, was verified by statistically comparing it to independent instrumental data. It indicates that the Fairbanks area has been warmer in the twentieth century than in the nineteenth century during these months. As the longest annual record of temperature from northern Alaska, this reconstruction provides quantitative evidence for a climatic warming similar to that occurring throughout large portions of the Northern Hemisphere during the past 100 yr (refs 10–12).

Fourteen ring-width chronologies were obtained according to standard dendrochronological techniques¹³ from sites (67–68° N, 153–155° W) within continuous forest and treeline environments in the Walker Lake–Alatna River valley area of the Brooks Range. Each chronology represents the average of age-standardized ring widths¹⁴ of two increment cores from each of 15 or more trees. To examine climate–growth relationships, the chronology of each site was used as the dependent variable in a stepwise multiple regression analysis with monthly climatic data from the Interior Basin climatic division¹⁶ within which the sites are located. This analysis, termed response function analysis by Fritts *et al.*¹⁷, defines the correlations of radial growth with monthly temperature and precipitation during and preceding the actual growing season. Months before the growth season are included in the analysis because climatic conditions during this period can affect growth during the subsequent summer¹⁵. The growth variance accounted for by temperature and precipitation in the 14 analyses ranges from 11.2 to 54.7% and averages 36.0%.

The basic form of the response function was similar at all sites and these results are summarized in Fig. 1. This set of climate–growth correlations suggests physiological processes which limit growth in the sub-Arctic. In addition to the expected positive

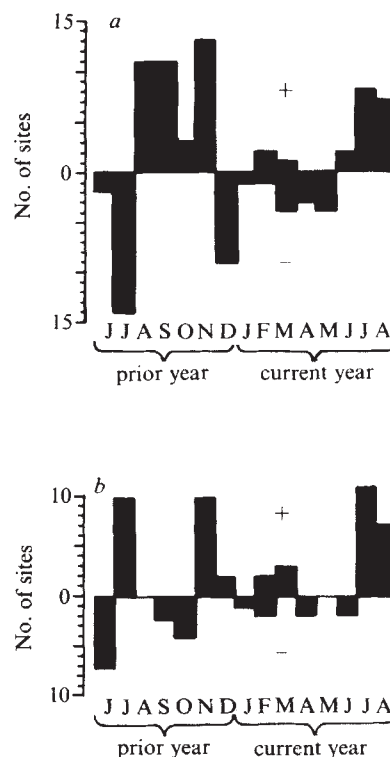


Fig. 1 Response function summary for: a, temperature; and b, precipitation. The vertical scale represents the number of sites exhibiting significant ($P < 0.05$) positive (upper half of scale) or negative (lower half of scale) correlation with each climatic variable. The climatic variables are mean monthly temperature and total monthly precipitation for the June before the growth season through August of the current year.

correlation with current summer temperature, growth exhibits a strong positive correlation with previous autumn temperature and negative correlations with previous summer, winter and spring temperatures. The positive response of growth to current summer temperature suggests that warm temperatures can increase rates of net photosynthesis and enhance growth in treeline environments during the growing season. The prominent positive correlation with previous autumn temperature suggests that warm autumn temperatures lead to increased photosynthesis after the end of the growing season, resulting in greater reserves of carbohydrates for the next year. Note that this correlation exists for periods after growth has ceased in the preceding summer, suggesting that photosynthates are put into reserve rather than used for late-season growth. In fact, climatic conditions that hasten the cessation of growth during one summer may actually promote increased growth during the next. This hypothesis is consistent with the negative correlation of growth at all sites with previous July temperature, as low temperatures near the end of the growing season can cause early termination of growth^{18,19}.

The negative correlation of growth with winter–spring temperature may reflect the adverse effects of winter desiccation^{19–21}. Thawing of foliage precedes thawing of the stem and soil during periods of above average temperature. As a result, transpiration is activated while water uptake is inhibited, leading to severe water stress. Coupled with this is an increase in respiration which, if prolonged, will lead to death of foliage²³.

While precipitation during certain months is strongly correlated with growth, the responses do not follow clear seasonal trends and are not as easy to interpret as the temperature responses. The water relations of high latitude trees, particularly during the dormant months, has not been studied extensively, making evaluation of these responses difficult. However, the positive correlation for current summer months suggests that internal water stress can limit photosynthesis at sub-Arctic treeline sites when temperatures are high. Relationships of this

type are commonly found on arid or semi-arid sites where moisture stress limits growth¹⁵. The south flank of the Brooks Range is, in fact, a dry environment, as it receives an average of only 332 mm of precipitation per year¹⁶.

These response function results suggest that ring-width sequences from Interior Alaska contain significant climatic information and can be used to reconstruct past climatic fluctuations. Dendroclimatic reconstruction is a multistep process developed over the past decade by Fritts and others^{6,17,24}. In this study, principal component analysis was performed on the tree-ring chronologies to concentrate the information present in the data into a small number of variables (eigenvectors) and to satisfy the statistical assumption that regression variables are linearly independent.

Annual values (amplitudes) associated with the first five eigenvectors served as the tree-growth data set in the reconstruction procedure. Numerical relationships between these amplitudes and selected Fairbanks climatic variables¹⁶ were defined by stepwise multiple regression analysis in a process termed calibration. Fairbanks, though nearly 400 km south-east of the study area, has the longest climatic record in the Interior Basin Climatic Division. In each regression equation, the monthly temperature or precipitation measurements were expressed in terms of the tree-growth eigenvector amplitudes for the period 1931–74. Climatic variables were reconstructed by entering eigenvector amplitudes for the period 1828–1932 into the regression equations. Finally, the reconstructions were

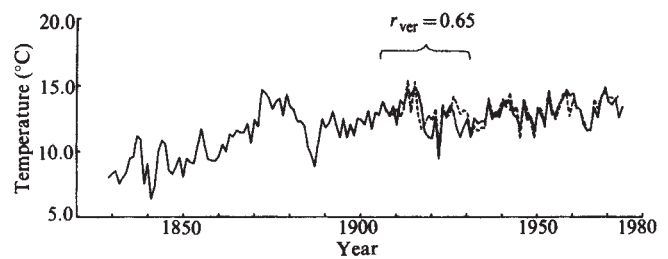


Fig. 2 Climatic reconstructions (—) and Fairbanks weather station data (---) for mean May–July temperature. r_{ver} is the correlation between the reconstructed and instrumental values for the verification period (1906–30).

verified by statistically testing their similarity to Fairbanks instrumental records for 1906–30 which had been withheld from the calibration. Reconstructions of May, June and July temperature passed ($P < 0.05$) two, three and four of the four verification tests respectively. The individual reconstructions of these months were averaged for a simplified reconstruction of early growth season temperature which passed three of the tests. February temperature and December precipitation each passed one of the verification tests, lending some validity to these reconstructions. However, this is not considered sufficient verification for climatic interpretation. These results, along with the R^2 values for the corresponding calibration equations are presented in Table 1.

The reconstruction of average May–July temperature (Fig. 2) indicates that the twentieth century has been approximately 2.1 °C warmer than the nineteenth century during these months.

This reconstructed warming episode is consistent with our knowledge of treeline fluctuations in Alaska. In the study area, healthy seedlings are presently abundant above the treeline, and upper elevation stands of white spruce are apparently not greatly stressed. These findings indicate a current advance of treeline, already noted in other parts of Alaska^{25,26}, and Scandinavia²⁷. The expansion of the boreal forest, coupled with the warming trend reconstructed for the past 100 yr, is evidence of the widespread warming which has been observed in other areas of the Northern Hemisphere during this period^{10–12}. The magnitude of the climatic warming also supports the finding that climatic fluctuations at northern latitudes tend to be more pronounced than simultaneous variations in temperate regions^{10,11}.

This study demonstrates the usefulness of Alaskan tree-ring sequences and climatic records for dendroclimatic reconstruction. In particular, climatic data from distant stations may be used successfully, so that sampling need not be restricted to those few areas near meteorological stations with long records. Our reconstructions can be extended at least 200 yr by the collection of longer chronologies from the Brooks Range. Refinement of the statistical models will give additional months of temperature and precipitation to the reconstructed data set producing a more complete view of past climatic fluctuations in northern Alaska.

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Table 1 Calibration and verification statistics

Climatic variable	R^2	Correlation	Sign test	Product mean	Reduction of error	No. of tests passed
February temperature	0.394	—	—	*	—	1
May temperature	0.654	*	—	*	—	2
June temperature	0.628	†	*	*	—	3
July temperature	0.475	†	*	*	‡	4
December precipitation	0.369	—	—	*	—	1
Average of reconstructed May–July temperature		†	*	*	—	3

—, Not significant; *, significant at $P < 0.05$; †, significant at $P < 0.01$; ‡, no significance test available; positive value considered significant (H. C. Fritts, personal communication).

The agreement between instrumental climatic data and the values predicted by the regression equation for the calibration period is reflected in the R^2 value. This statistic can be interpreted as the percentage of the climatic variance accounted for by the tree-ring data. The regression model included the amplitudes of the first five tree-ring eigenvectors, which account for 90.8% of the tree-growth variance as independent variables in the regression. Eigenvectors beyond five account for little variance individually and are assumed to represent random variation. Additional predictive power was gained by lagging these amplitudes 2 yr behind and 1 yr ahead of the climatic data for 20 possible independent variables (five eigenvectors at four lags). The final regression equation is

$$y_t = (b_1)(x_{1,t}) + (b_2)(x_{2,t}) + \dots + (b_{20})(x_{20,t})$$

where y_t is the predicted value of mean monthly temperature or total monthly precipitation for $t = 1932–72$. (Here, if June–August represent the growing season of year t , the preceding nine months are also considered to be in year t , even though September to December are actually part of the preceding calendar year.) b_i is the regression coefficient for the amplitude $i = 1, 20$, and $x_{i,t}$ is the amplitude value for $i = 1, 20$ and $t = 1932–72$. Again, the 20 coefficients for year t correspond to the amplitudes of eigenvectors 1–5 for years $t-1$, t , $t+1$ and $t+2$. In the stepwise multiple regression, only those variables with an F ratio > 1 were used as predictors in the final equation. Four verification tests were performed on each reconstruction to evaluate the correspondence of reconstructed values with actual climatic data. (1) The correlation test is a standard measure of the predictability between reconstructed and actual climatic data before the calibration period. (2) The sign test is a nonparametric procedure which counts the number of agreements and disagreements in sign changes between adjacent years in the two series. (3) The product mean assesses both the sign and the magnitude of the departures from the calibration period averages. (4) The reduction of error (RE) is a very rigorous test of association between a series of actual values and their estimates. It compares the sum of squares of the differences between the reconstructed and actual data with the sum of squares of differences between the actual data and their mean. The degrees of freedom used to test the significance of the correlation and product mean tests were adjusted downward to correct for serial correlation present in the reconstructed and instrumental time series²⁸. These tests are described in detail elsewhere¹⁵.

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Observations of dilatancy-induced polarization anomalies and earthquake prediction

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Much of the search for earthquake prediction schemes has centred on the expectation that rocks in the impending source area will show dilatancy: the opening of cracks at high stress-levels. Recent developments in the theory of seismic wave propagation in cracked media¹ demonstrate that shear-wave splitting will occur in seismic-wave propagation through cracked structures, when the cracks have any overall alignment. This splitting causes polarization anomalies in the seismograms, and it has been suggested¹ that dilatancy, induced by the build-up of stress before earthquakes, might be recognized and monitored by analysis of these anomalies. We present here new observations which support this hypothesis.

The crust and upper mantle of the Earth are thoroughly permeated by cracks. Sedimentary rocks contain joints and fractures, which can lead to pronounced seismic velocity-anisotropy in shallow beds^{2,3}. Igneous rocks in the crust, and possibly the crystalline upper-mantle⁴, contain microcracks within grains and along grain boundaries⁵. These various cracks are essentially closed and transparent to seismic waves under high isotropic confining-pressure and low pore-pressure⁴. The primary action of non-hydrostatic stress on rock will be to modify the existing lines of weakness (cracks) in the rock, and the effects will depend on whether the cracks are initially open or closed, and on the relative confining and pore-pressures. At low ambient-stress and low pore-pressure, systems of open cracks may be differentially closed⁶, and at high ambient-stress, systems of closed cracks may be differentially opened, if the pore pressure and non-lithostatic stresses are sufficiently high. The term 'dilatancy' is used here to cover both these differential processes, in addition to the usual meaning of the opening of fresh cracks under

high local stress-levels. This differential opening of existing cracks may occur over substantial distances, and is probably the direct cause of many of the precursory phenomena, which are sometimes observed hundreds of kilometres from impending epicentres⁷. Laboratory experiments demonstrate that, in stressed rock-samples, existing cracks⁶ and newly opened cracks^{8–10} both take up pronounced alignments, and certainly all stress-induced dilatancy in the Earth will open cracks in preferred directions. Note that there is now a hypothesis that gas at high pore-pressure may exist at deep levels in seismic regions¹¹, so that dilatancy cracks could be gas filled even in the upper mantle.

The wavelengths of seismic waves are so much greater than the likely dimensions of any stress-induced cracks, that the velocity variations of seismic waves propagating through a dilatancy zone of aligned cracks can be modelled by propagation through purely-elastic anisotropic media¹. Theoretical and numerical calculations demonstrate^{1,12,13} that, except in a few specific symmetry-directions, anisotropy writes a characteristic signature into the polarization of the shear wavetrain. This signature is a distinctive feature of seismic waves propagating through anisotropic media and will persist for any following isotropic-segments of the path. A plane shear-wave splits on entry into a dilatancy zone into two orthogonally-polarized phases, which are not, in general, parallel to either the vertical or horizontal directions (curved wavefronts show more complicated effects). These split waves travel with different velocities, and result in the shear-wave particle-motion being resolvable into nearly-orthogonally polarized components^{1,13}. The arrival of these 'split' shear-waves may be identified by abrupt changes in direction (anomalies) of the particle motion, when plotted in polarization diagrams¹. The time delay between the phases may be estimated from suitable rotated seismograms, or more directly from polarization diagrams. This delay is proportional to the degree of differential shear-wave anisotropy, and the length of the path through the anisotropic rock, which for long paths will be the dimensions of the dilatancy zone. Consequently, the delay is not very sensitive to small errors in epicentral location or focal depth. However, it is sensitive even to quite weak crack-anisotropy¹, and seems to be a way of monitoring the whole dilatancy episode from initiation of the stress-induced cracking, through the possible influx of water¹⁴, to the conclusion of the earthquake sequence. An example of the sensitivity is that saturation of dry cracks by water would approximately double the differential shear-wave anisotropy for about half of all directions of propagation through any given system of cracks¹. This should be recognizable on the seismograms, and may help to resolve whether cracks are gas filled at depth in the crust, and whether the influx of water is an essential part of the source process. Thus, if they can be observed, shear-wave polarization-anomalies could lead to a better understanding of the whole earthquake source-process.

We believe that such anomalies have now been observed. For several years, delays between shear-wave arrivals on pairs of unrotated horizontal-component seismograms have been recorded¹⁵ on arrays of three-component seismometers in seismic regions in Armenia, and Fergana, USSR. The delays were observed on between 5 and 10% of all seismograms from local and regional events at all azimuths and at distances up to 450 km. These delays were interpreted¹⁵ as being due to shear-wave splitting caused by anisotropy directly induced by the build-up of stress before an earthquake. Recently, the magnitudes of these delays have been shown to vary before large earthquakes, and to be correlated with the directions of nearby geological faults¹⁶. However, we consider that anisotropy due to crack dilatancy is likely to dominate any anisotropy directly induced by stress in the Earth.

Gupta¹⁷ probably observed similar anomalies to those of Yegorkina *et al.*¹⁵ from several series of earthquakes in Nevada, US, at distances of >100 km from a single three-component seismic station. However, the small range of azimuths did not allow any convincing interpretation¹⁸, and Gupta was misled

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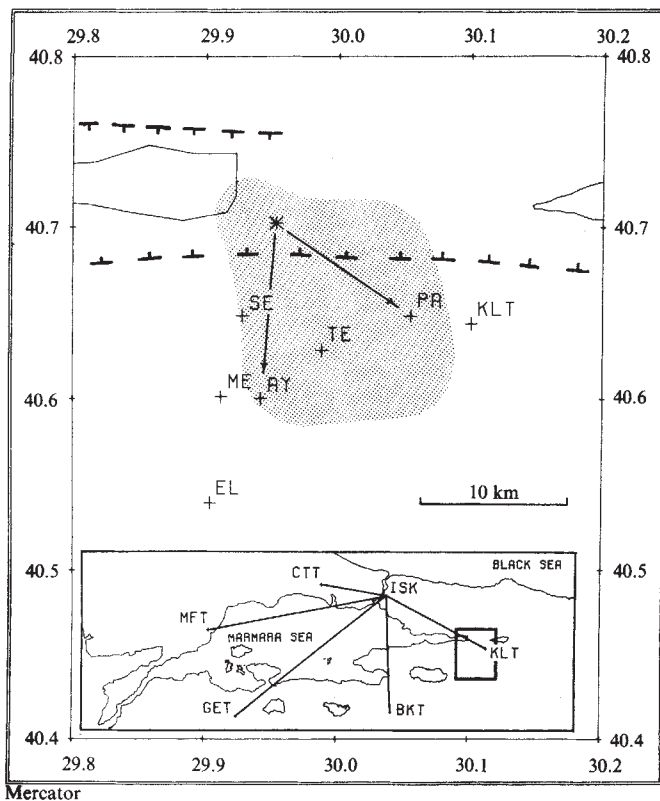


Fig. 1 Map showing the TDPNET three-component stations, with inset of MARNET stations. Most of the local earthquakes occurred within the shaded area, and the epicentre of the earthquake used for Fig. 2 is marked by an asterisk. The heavy dashed line shows the approximate lines of the Northern Anatolian Fault, which in this area appears to be a graben structure.

into seeking shear-waves split only into SV and SH components, when the seismic traces clearly did not display such polarizations. Gupta's observations will be discussed in detail elsewhere.

Similar delays have been observed on records from a small closely-spaced network in Turkey, TDPNET (Fig. 1), of three-component seismometers specifically designed to monitor polarization anomalies. A centre of low-magnitude seismic activity was identified near the Northern Anatolian Fault from MARNET records (MARNET is a radio-linked seismic network spanning the Marmara Sea, Turkey). A collaborative project between the Institute of Geological Sciences and Kandilli Observatory deployed TDPNET over the swarm activity for eight weeks during the summer of 1979. High-quality recordings were obtained from several hundred small earthquakes at epicentral distances of <20 km, and depths between 8 and 15 km. The analogue magnetic-tape records were digitized at $100 \text{ samples s}^{-1}$, and display impulsive P- and S-wave arrivals very suitable for polarization analysis. A few records show shear waves arriving at different times on the unrotated horizontal-seismograms from events at all azimuths, similar to the observations of Yegorkina *et al.*^{15,16}. The majority, however, show shear waves arriving at the same time on both horizontal traces, and display shear-wave splitting only when plotted in polarization diagrams.

Figure 2 shows examples of both types of record from the same earthquake at different stations. The seismograms are typical of the data set: almost every shear wavetrain with sufficient signal-to-noise ratio (several hundred examples) shows shear-wave splitting in polarization diagrams, and a few show splitting in the unrotated horizontal-seismograms, as in the observations of Yegorkina *et al.* Figure 2a shows separate shear-wave arrivals on the two horizontal-components, despite

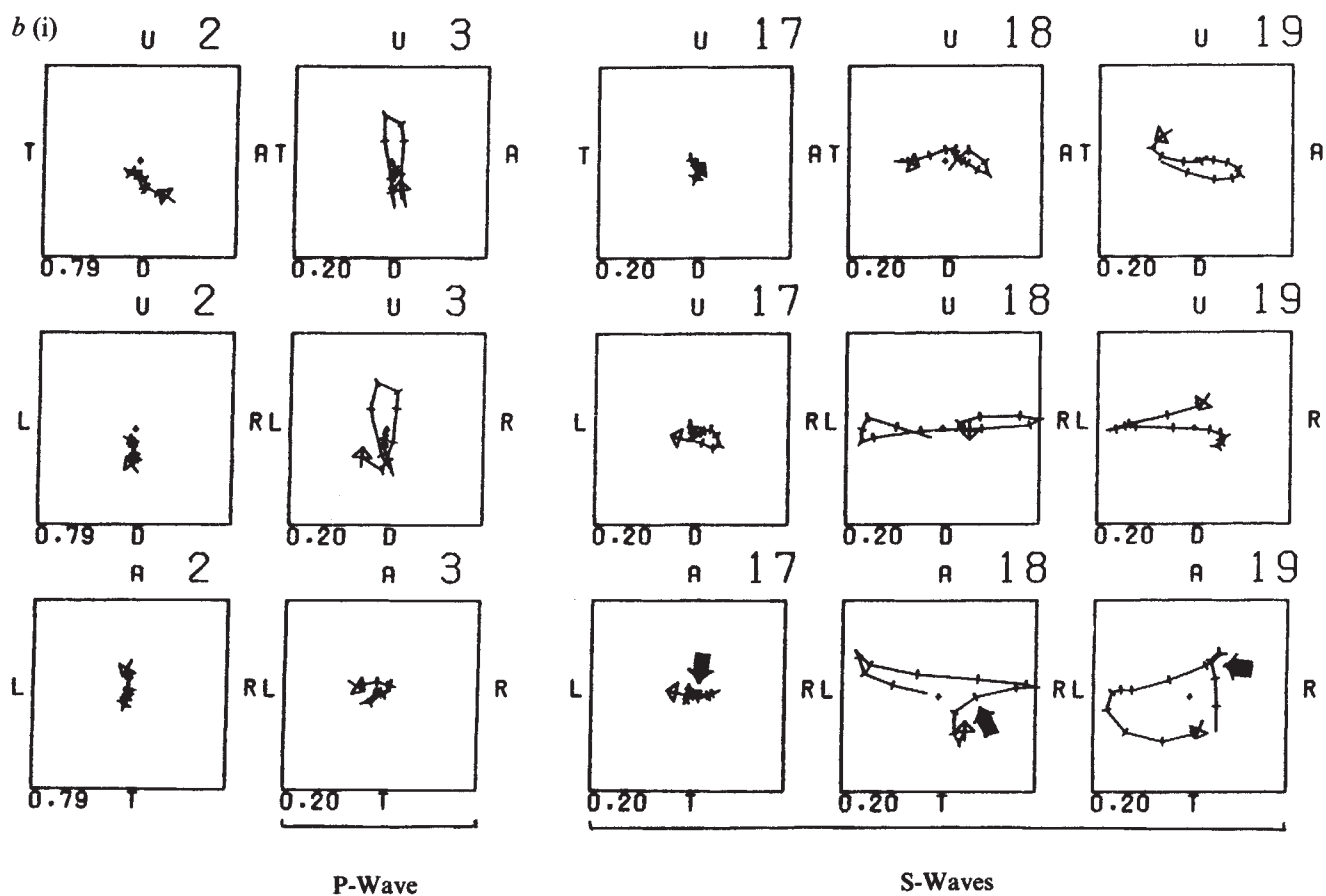
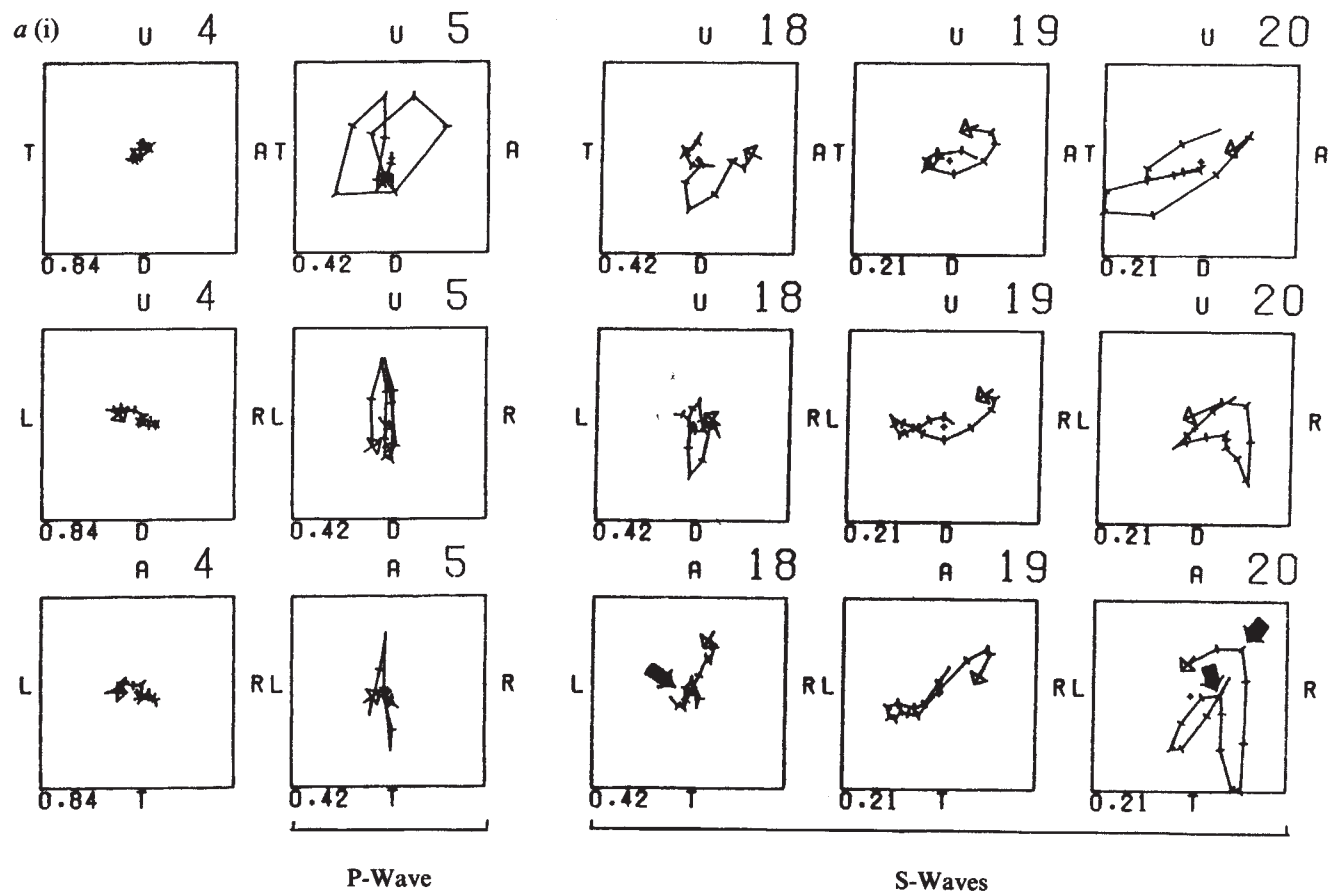
the azimuth to the epicentre being 40° from the instrument orientations: the shear arrival on the east-west component is about 0.2 s later than the arrival on the north-south. Figure 2b shows shear waves arriving simultaneously on both horizontal components. Splitting is not easily identified on the original or the rotated seismograms, but is clearly visible in the horizontal projection of the polarization diagrams.

The horizontal polarizations diagrams show abrupt changes in the direction of the particle motion. We interpret these anomalies as the arrival of phases with different linear polarizations due to shear-wave splitting in propagation through anisotropic material. Any other explanation is untenable: the anomalies cannot be caused by isotropic discontinuities, because no isotropic structure can split shear waves propagating in almost all directions; and it is unlikely to be a source effect, when the sources are small and the P-wave arrivals comparatively simple. There are very few previous studies of short-period particle-motion so it is difficult to know what sort of phenomena to expect. Local and regional earthquakes that we have examined in a region of low seismicity (Scotland) have elliptical shear-motion without the characteristic abrupt turning points of the TDPNET records. Orthogonal changes in polarization are sometimes observed in local and regional events, when there is a mixture of Rayleigh and Love surface-waves present. However, this cannot account for orthogonal polarizations in the first half-second following the initial shear-arrival, particularly when the paths are direct rays with little possibility of head-wave, channel-wave, or surface-wave interactions. We consider that some form of anisotropy must be the cause of the split shear-arrivals.

We suggest that dilatancy is the only possible explanation of this anisotropy. Stress on cracked rock (all crustal rocks are heavily cracked^{2,5}, although many of these cracks may be initially sealed) causes the cracks to open and close anisotropically⁶, and such dilatant structures are expected to cause shear-wave splitting¹. Dilatancy is the only possible explanation of anisotropy, which can have such large, if not dominating, effects in such otherwise heterogeneous regions as Turkey and Armenia. A demonstration that the delays between the shear waves vary with time (stress) would confirm our interpretation.

Clearly, such splitting is not easy to analyse. However, some conclusions can be drawn from the existing data: many of the shear waves in the TDPNET records (Fig. 2a) and Armenia¹⁵ do not split parallel to SH or SV, but into more general orientations. This supports the hypothesis that the splitting is not caused by isotropic conversions. It also suggests that the dilatancy is unlikely to consist of horizontal cracking as suggested by Wang¹⁹, which would result in transverse isotropy and splitting only into SH or SV components.

Figure 2 shows several abrupt changes in direction of the shear-wave polarizations. These may be due to complicated local structure, but may also be due to a curved wavefront propagating through a structure with comparatively strong anisotropy. The group-velocity vector is not, in general, parallel to the phase-velocity vector in anisotropic media¹². Consequently, the map of the positions reached by the energy of two shear-waves radiating from a point in a given time, the wave surface, may be extremely complicated, with three-dimensional cusps, ridges, and multiple points. Cusps, in particular, are very common features of shear-wave energy propagation in anything other than very weak anisotropy. Rays propagating in directions within a cusp may have four shear-wave arrivals, with different velocities, different polarizations, and wavefronts which are not parallel. The density of cracks in Earth structures is large enough to produce strong anisotropy: the only detailed field measurements of velocity anisotropy known to us² were interpreted³ as due to two sets of intersecting cracks, each with crack density of the order of 0.2. The large crack densities were due to large dimensions and aspect ratios. Thus, some crack anisotropy in the Earth is strong enough to produce complicated wave-surfaces containing cusps, and this is preferred explanation for the several abrupt changes of polarization in Fig. 2.



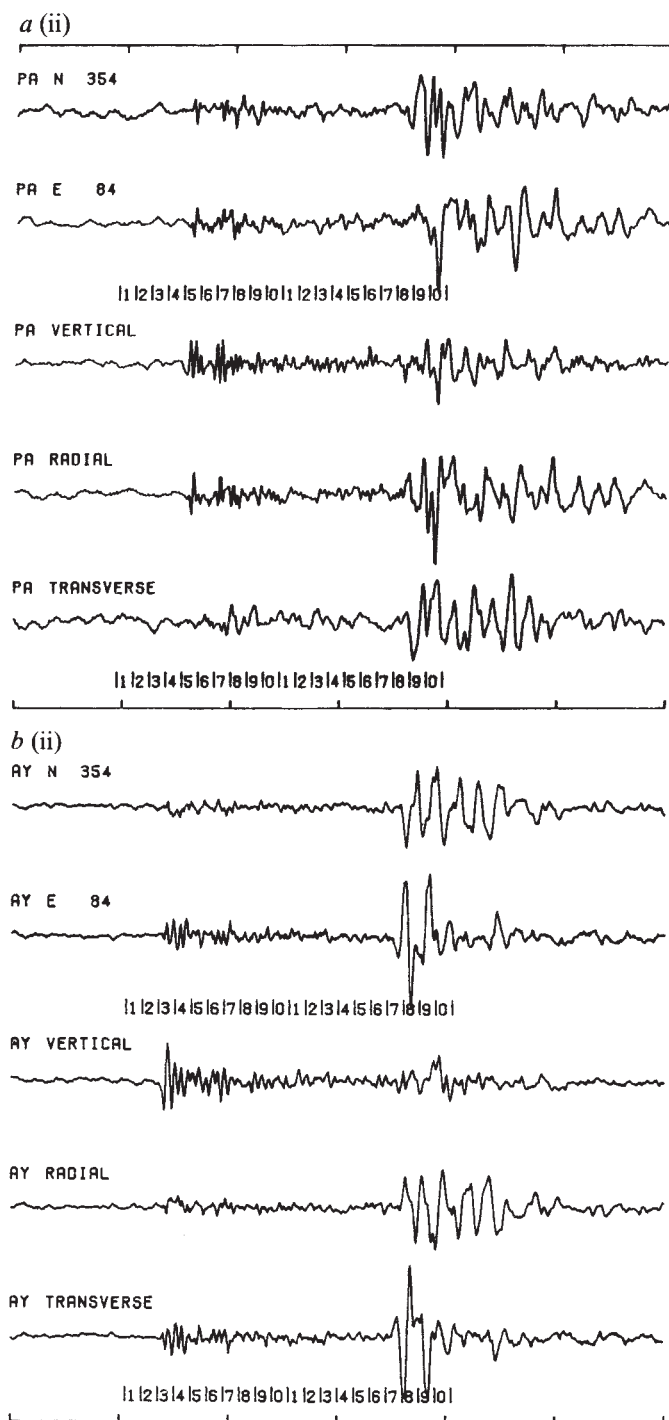


Fig. 2 Examples of seismograms and polarization diagrams for a small earthquake on 15 September 1979 with epicentre $40^{\circ}42'10''$ N, $29^{\circ}57'13''$ E, depth 13 km, and magnitude $M_L = 1.5$. The seismograms are, from the top: unrotated horizontals N 354° E and N 84° E, vertical, and rotated radial and transverse components. The numbered polarization-diagrams for P- and S-wave arrivals are the projections of the particle motion on to three orthogonal-planes for the correspondingly numbered 0.15-s time-windows marked either side of the rotated traces. Directions: U, up; D, down; T, towards; A, away from the source. L, left; R, right in the direction of the station. Each set of three diagrams has been normalized and the relative multiplication factor is marked at the bottom left of each diagram. The heavy arrows on the horizontal projection of the S waves mark the first S-wave arrival, and the subsequent arrivals of phases with different polarizations. Cross bars marked on the polarization diagrams at every 1/100 s, allow delays to be estimated. *a*, Seismograms at PA: hypocentral distance 17 km; azimuth of arrival N 305° E. *b*, Seismograms at AY: hypocentral distance 18 km; azimuth of arrival N 5° E.

Our comparatively simple hypothesis of extensive dilatancy links several previously unrelated earthquake phenomena, and seems to be present to the several seismic regions of North Anatolia, Armenia, Fergana, and possibly Nevada. Our suggestion that stress in the Earth can be monitored by examining shear wavetrains in polarization diagrams would be very important for earthquake prediction studies. All previous precursors, including V_p/V_s anomalies¹, are probably indirect effects of this dilatancy, and seem to occur unpredictably. The TDP experiment suggests that polarization anomalies may occur in almost all shear wavetrains propagating through dilatancy zones, and might be generally available for estimating changes in the Earth's stress.

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Tritium in the deep North Atlantic Ocean

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A distinct core of tritium (from nuclear weapons testing in the atmosphere) marks the southward flowing jet of the deep western boundary current of the North Atlantic circulation at 30° N latitude. The concentrations, of the order of 0.5–1.0 TU (1 tritium unit = $1\text{ }^3\text{H}$ atom per $10^8\text{ }^1\text{H}$ atoms), indicate a roughly 10-fold dilution of source waters in the far north. This thin jet, 50 km in width, carries water into subtropical regions which, until recently, were free of anthropogenic tritium. It underlies the more extensive distributions of tritium found in more rapidly renewed waters above the permanent thermocline.

The deep waters of the world's oceans have descended from the sea surface in a very few regions of the far northern and southern Atlantic. Stable density stratification constrains the actual sinking processes to be locally intense and variable, but the great mass of deep ocean is such that the replenishment process proceeds on a time scale of centuries. This planetary-scale thermohaline convection thus provides a long time-constant for many aspects of the ocean-atmosphere system.

Both climatic change and the dispersal of pollutants and trace chemicals in the biosphere are influenced by the slow overturning of the sea.

Observations¹⁻⁵ (Fig. 1a) and theory⁶⁻⁷ suggest that the deep and abyssal flow proceeds initially equatorwards, away from the sources of sinking water, along concentrated paths lying near western continental or topographic boundaries. A complementary poleward and upward circulation occurs, apparently, as a broad, weak flow in the interior of the ocean basins. The combination of this circulation with turbulent exchange between boundary current and interior establishes the large-scale

gradients of chemical properties, such as the saline tongue⁸ in Fig. 1a. The flow at this temperature horizon (1.9 °C) is traceable to both Arctic and Antarctic sinking regions.

The decisive influence of boundary currents on the global distributions of trace material has motivated an observational programme, described elsewhere⁹, at a site where the deep boundary current is particularly well defined, Fig. 1b. In this programme current meters, CTD profilers, XBT probes and, by chance, visitation, drifting surface- and deep floats were used to examine the core of the current, and its relationship with the basin-wide circulation.

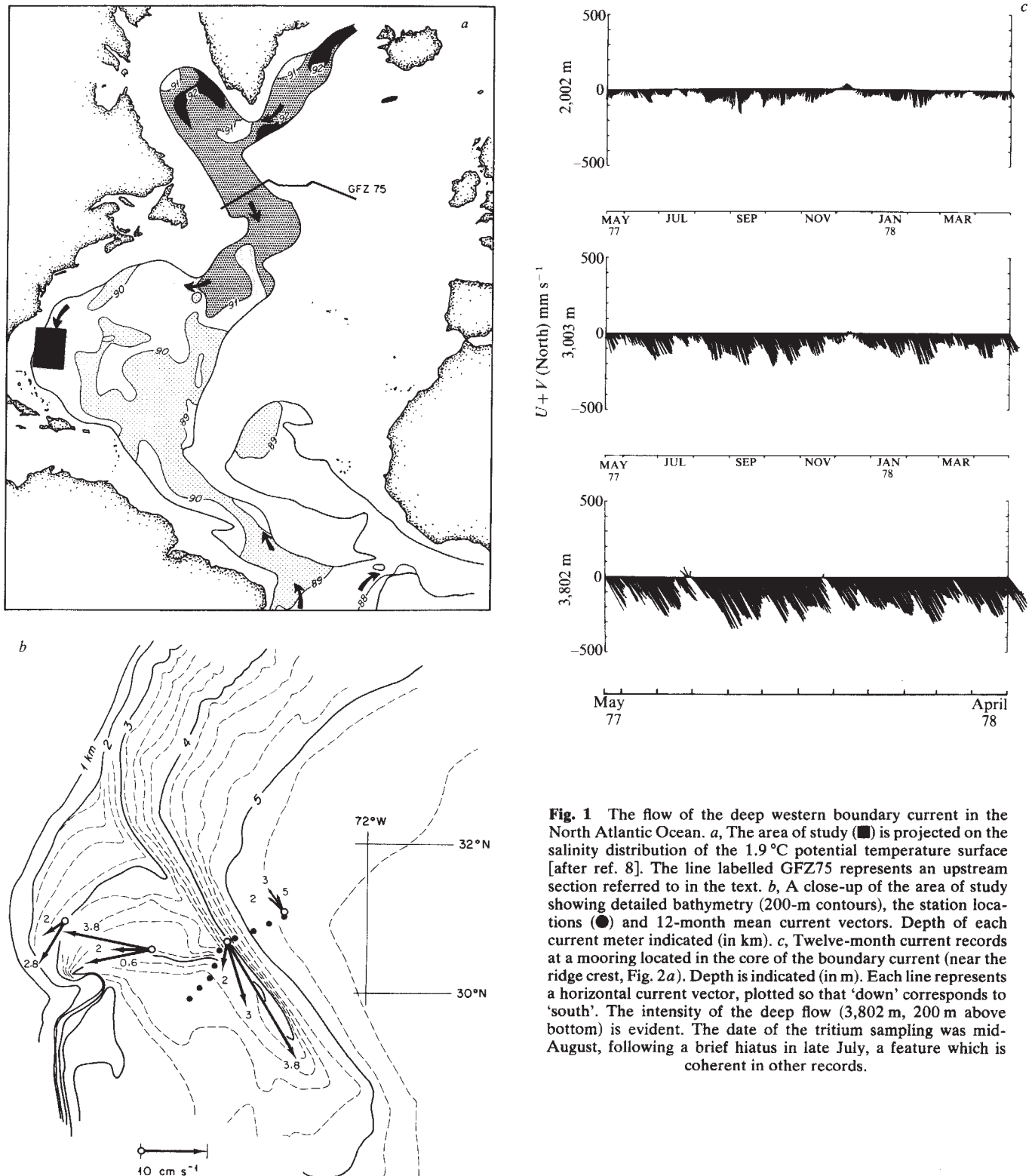


Fig. 1 The flow of the deep western boundary current in the North Atlantic Ocean. *a*, The area of study (■) is projected on the salinity distribution of the 1.9 °C potential temperature surface [after ref. 8]. The line labelled GFZ75 represents an upstream section referred to in the text. *b*, A close-up of the area of study showing detailed bathymetry (200-m contours), the station locations (●) and 12-month mean current vectors. Depth of each current meter indicated (in km). *c*, Twelve-month current records at a mooring located in the core of the boundary current (near the ridge crest, Fig. 2a). Depth is indicated (in m). Each line represents a horizontal current vector, plotted so that 'down' corresponds to 'south'. The intensity of the deep flow (3,802 m, 200 m above bottom) is evident. The date of the tritium sampling was mid-August, following a brief hiatus in late July, a feature which is coherent in other records.

As a part of the geochemical programme, 280 samples were taken for tritium analysis. Being part of the water molecule, tritium represents an ideally passive tracer. There are no known chemical or biological processes in the deep sea that alter or scavenge it. Tritium has well-defined but time-dependent boundary conditions and its lifetime (12.26 yr half life) is comparable to the circulation time scale of the western deep North Atlantic. Unlike the relatively steady-state nature of other geochemicals, the natural inventory of tritium has been dwarfed by massive production by recent nuclear weapons testing¹⁰. This tritium 'spike' entered the surface oceans primarily at high northern latitudes^{11,12} in the form of tritiated water. Natural surface water tritium concentrations were about 0.2 TU. Present subtropical surface water concentrations are of the order of 5 TU, and were higher than this in 1964–65, soon after the peak in weapons testing. Ostlund *et al.*¹³ reported tritium measurements from the 1972 GEOSECS expedition which clearly showed the sinking and southward penetration of tritium. South of 40°N, however, the North Atlantic GEOSECS track left the western boundary, and no further abyssal tritium was apparently recorded. At shallower depths other studies have documented the downward penetration of tritium into the main thermocline^{14,15}.

In the present experiment we sought to determine how much water flows equatorwards at the mid-latitude western boundary, and how much of this water is of far-northern origin. The observations were sited near the Blake–Bahama Outer Ridge at 30°N latitude. This prominent sedimentary ridge acts as the western boundary of the ocean at levels deeper than ~3,500 m. The 12-month average currents seen at four moorings show clearly the boundary current winding along contours of constant depth (Fig. 1b). The easternmost mooring exhibits a weak flow counter to the jet (northwestward), thus establishing the width of the jet at ~50 km.

A sample current record⁹ (Fig. 1c) shows the increase in intensity of the jet with depth, on the eastern flank of the ridge. Despite surges of 40 cm s⁻¹ and occasional periods with no southward flow, the mean velocity, 21 cm s⁻¹, is quite representative. The time of tritium sampling, 4–8 August 1977, occurred shortly after a hiatus, but is characterized by velocity typical of mean conditions.

Samples were taken using a 24-bottle, 1.2-l Niskin rosette sampler mounted on the CTD frame. The samples were transferred to GEOSECS-type clamped copper tubes¹⁶. Tritium was determined by the ³He regrowth technique¹⁷ to an accuracy of 1.5% and a detection limit of 0.04 TU.

The deep western boundary current is distinctly outlined by a high concentration of tritium, Fig. 2a. The core overlies the ridge at ~4 km depth. Tritium levels decrease dramatically both above and to the east of the jet, but with some evidence of activity in the westernmost part of the section. The potential isotherms (dashed) superimposed on Fig. 2 are banked up on both sides of the ridge, consistent with the strong southward flow on the eastern side, and northward flow on the western side.

The most intense (highest tritium level) part of the core is noticeably smaller than the region of banked isotherms. The tritium isopleths extend over the ridge-crest, more or less along potential isotherms. If this were an ideal flow directly parallel to the depth contours, the currents on the western and eastern sides of the crest would be effectively 400 km apart, in the downstream sense. The continuity of the tritium core, however, suggests that some cross-ridge flow is taking place. This flow is verified both in current-meter and drifting float data (note the veering with depth of the mean current vectors, Fig. 1b). The eastward turn in the depth contours is apparently too sharp to be negotiated by the jet, which rides over the crest.

The tritium data are plotted against potential temperature in Fig. 2b. For reference we have also plotted the mean data for the *Panulirus* station off Bermuda (32°N, 64.5°W)¹⁵, and for a section across 53°N¹⁸. This section is labelled GFZ-75 on Fig. 1a. The *Panulirus* data were taken at essentially the same time as the present section, whereas the 53°N section occurred 2 yr

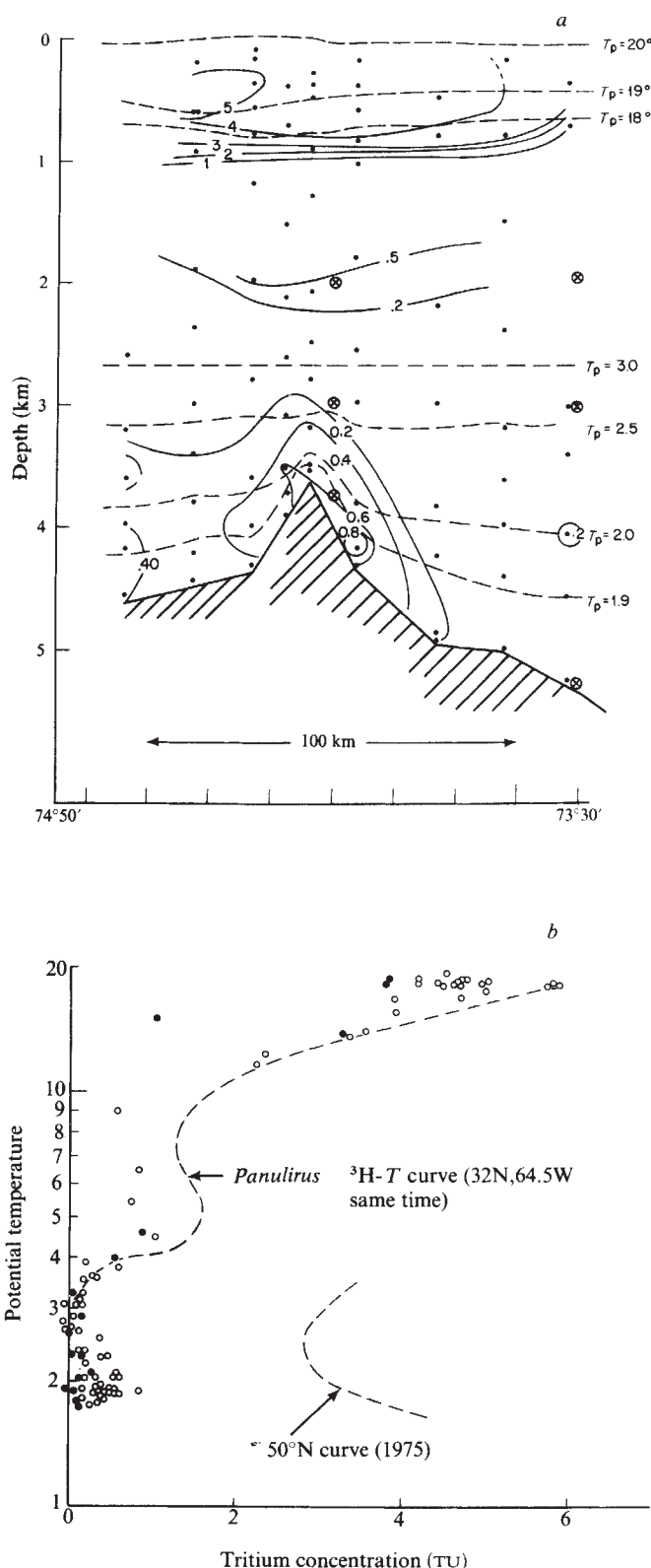


Fig. 2 The tritium results for the section. *a*, The tritium isopleths (solid lines) for the undercurrent hug the ridge, and extend over the ridge in a manner parallel to the isotherms (dashed lines). The far-northern water, marked in this way, occupies only a small part of the broader southward flow at this time. Note also the westward enhancement of the shallow (350 m) tritium maximum, and the associated thermal structure (that is, the dip in the 18 and 19°C isotherms). ⊗, Current meter positions. *b*, The temperature-tritium relation for the stations. ●, Stations outside of the current; ○, corresponds to undercurrent stations. For comparison, the T_P - 3H curves for a central Sargasso Sea station (*Panulirus*) and an upstream section (see Fig. 1a) are shown.

earlier. The principal source for the deepest levels is the Denmark Strait overflow of waters from the Greenland–Norwegian Sea, with lesser contributions from the Iceland–Scotland overflow farther to the east. If the far northern section indeed represents the upstream boundary conditions for the deep flow at the Blake–Bahama Outer Ridge, then we are observing an effective dilution of those waters by a factor of 5–10.

Above the tritium core the concentrations decrease essentially to zero at $\sim 2.8^\circ\text{C}$ or 3,000 m depth (0.01 ± 0.03 TU, seven analyses). It is remarkable that this zero layer occurs at these temperatures, for a persistent southward flow is observed at 2,000 m and below (Fig. 1b), and the penetration of North Atlantic Deep Water at these levels throughout the world ocean requires southward transport at this latitude. At the time of this section, the water in the 'zero layer' had apparently been entrained from subtropical mid-ocean, rather than having flowed simply from the far north. SOFAR float data of Rossby exhibit several chance occurrences of this kind of entrainment at 1,900 m depth. An upper limit to the amount of 'northern component water' at this depth is found by comparing the <0.07 TU (95% confidence limit) in this water with the >2 TU found in the corresponding source waters, giving a maximum contribution of a few per cent. The dynamics of the mean circulation changes greatly with depth, as shown by this cut-off of tritium. The role of mesoscale eddies may be important, both in determining the time-mean circulation¹⁹ and in causing sampling problems in sections like this one.

Above the 'zero layer', at temperature $>3.4^\circ\text{C}$, the tritium increases to a slight maximum in the range $4\text{--}6^\circ\text{C}$. This feature corresponds closely to the feature observed in the *Panulirus* data¹⁵, but it is less intense. The two data sets merge below this maximum ($3\text{--}4^\circ\text{C}$) and also within the main thermocline ($10\text{--}15^\circ\text{C}$). In the upper thermocline the mean gyre is larger in extent, and eddy stirring more intense, than in the deep water. One expects tritium to be more broadly distributed, therefore, at upper levels, just as Fig. 2b suggests. Above 18°C the present data tend to drop to lower tritium concentrations. The subsurface maximum is evidence of southward penetration of freshly ventilated 18° -water away from its formation region, just south of the Gulf Stream. A westward tritium enhancement can be seen in section in Fig. 2a, at a depth of 300–500 m. Analysis of the current meters and XBT data⁹ associates this upper pattern with a particularly large, energetic, anti-cyclonic eddy of the 18° -water. It may be that upper-level Gulf Stream rings and other thermocline eddies carry distinct tritium markings.

The detection of a substantial tritium signal in the core of the deep western boundary current at 30°N establishes a transit time of ≤ 15 yr for water from the far northern ocean surface, with from 5- to 10-fold dilution. The transit time along the deep western boundary, itself, would require only of the order of 2 yr, at speeds of the order of 10 cm s^{-1} . Abyssal tritium has not, to our knowledge, been seen before equatorwards of the polar front. Because of the eddy mixing and eddy-induced deep circulation, particularly that beneath the Gulf Stream, we could not be certain that the core of northern water would occur in the boundary current. In fact, a neighbouring section, 150 km to the south, showed far weaker anomalies of dissolved silicate, implying that other markers of northern origin like tritium would, in that instance, be weak as well. Conversely, we expect tritium to appear intermittently outside of the boundary current, in mid-ocean (although none was reported at this latitude, at 50°W longitude, in the GEOSECS section¹³).

The strong differences in eddy stirring, mixing, and mean circulation, from one depth to the next, are mirrored in the tritium concentration. Further sampling may help to establish the broader patterns of penetration across the Gulf Stream front. The results reported here underline the value of combining a variety of measurement techniques (current meters, XBT and CTD transects, floats and standard geochemistry) with radiochemical measurements. It would be of great import to problems in deep-water circulation and to the related problems in climatology (through oceanic heat-flux), atmospheric chem-

istry and dynamics, to obtain more such data before the tritium transient expires.

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New constraint on the maintenance of Mn nodules at the sediment surface

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Investigations into the association of manganese nodules with pelagic sediment in many areas of the deep ocean have mainly considered either (1) the source of metals in nodules, or (2) the occurrence of nodules predominantly at the sediment surface. The second problem is discussed here. The mechanisms previously proposed have failed to consider that nodules retain their orientation at the sediment surface for several hundred-thousand years, during which time several tens of centimetres of sediment are fluxed down beneath them. Although we have no conclusive evidence, we consider the most plausible explanation for the surface occurrence of nodules to be bioturbation by infauna.

Approximately half of all nodules occurring in the upper few metres of pelagic sediment are at the sediment surface^{1,2}. Radiometric and non-radiometric techniques^{3–5} require that these nodules accrete at a rate of $\sim 1\text{--}10\text{ mm Myr}^{-1}$. The fine-grained fraction of sediment on which nodules rest, consisting mostly of lithogenic debris and biogenic SiO_2 and CaO_3 , accumulates⁵ at a rate of $\sim 1\text{--}10\text{ mm kyr}^{-1}$. Why then are nodules not buried by this 'avalanche' of fine-grained sediment?

Nodules, usually spherical to discoidal, have a diameter between ~ 5 and 100 mm, but average⁷ about 30 mm. Assuming an approximate growth rate of 5 mm Myr^{-1} , which is independent of size⁸, nodule ages are of the order of 1–10 Myr. In most areas, the surface sediment on which they rest is still accumulating. What is the mechanism which redistributes the continuously accumulating fine-grained fraction of sediment beneath nodules? In some exceptional areas, however, nodules rest on ancient sediment⁹ where erosion may currently be occurring.

Because nodules are denser than the underlying sediment, this suggests that nodules should sink through the upper layer of unconsolidated clays, which consist¹⁰ of 80%, or more, interstitial water. Hypotheses explaining the surface occurrence of nodules have been coupled with explanations of how nodules

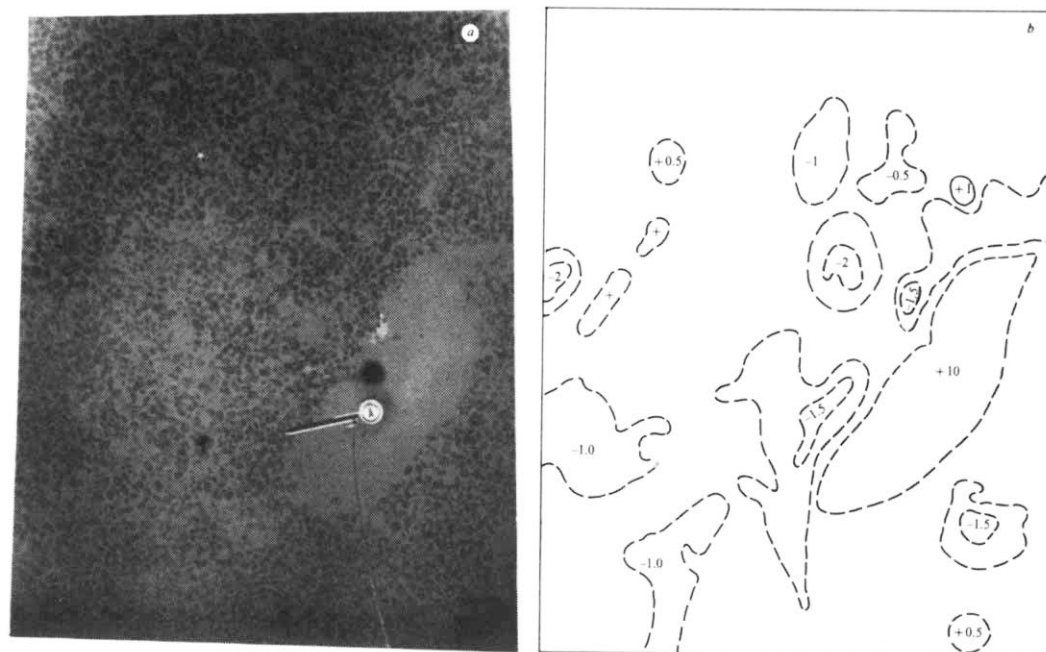


Fig. 1 *a*, Bottom photograph taken at 126°05' W, 15°08' N, 4,520 m depth. *b*, Relief map of the sea floor. The numbers in the enclosed contours give the approximate elevation (+) and depth (-) of these areas from the intervening area. The compass face is 7.5 cm in diameter. The compass is approximately 1 m above the bottom. Nodule coverage is often greatest in depressions; see, for example, the 2-cm depression north of the compass vane. The south-facing slope of this depression has low nodule coverage, suggesting that nodules have moved down slope into the depression. The area immediately surrounding the 10-cm mound has a nodule coverage similar to the rest of the area, suggesting that nodules have not been moved laterally during its construction.

are pushed up against this apparent tendency to sink. This approach requires active means of moving nodules upwards. There are several objections to these active mechanisms of keeping nodules on top of the sediment, but first we consider density.

According to Archimedes' principle, an object wholly or partially immersed in a fluid floats if its density is less and sinks if its density is greater than the fluid density. As the wet densities of nodules and sediment are ~ 2.4 and 1.3 g cm^{-3} respectively, why do nodules not sink through the upper layer of sediment? This layer, consisting of the most recently fallen sediment, is unconsolidated.

Vane shear measurements show that this sediment layer has solid- as well as fluid-like properties. Comparing these measurements with calculated loads exerted by nodules indicates that the sediment strength is much greater than that required to support surface nodules. Measurements have been made on box cores from the three Domes areas in the equatorial North Pacific¹⁰. Only the heaviest nodules and weakest sediment were considered. At a depth of 7 cm, the sediment has a strength 40–170-fold greater than that required to support surface nodules (Table 1). Shear strength measurements on the upper few centimetres of sediment are rare but they suggest a

decrease towards the surface of $\sim 75\%$. However, in the upper 7 cm of sediment, where nodules are most abundant, the critical load, the total load that sediment is capable of supporting before its failure, is six times greater than the measured shear strength, owing to the shear pattern developed during failure¹¹. Thus, once nodules are positioned at the surface, the sediment strength is more than adequate to support them, that is, to prevent their sinking.

Previous explanations of the sea floor occurrence of nodules can be divided into two groups of mechanisms, physical and biological. The physical group considers that nodules are picked up and/or rolled over by bottom currents¹², or maintained at the surface by seismic activity¹³. Glasby¹² considered the force that bottom currents might apply to a nodule and found that it is comparable with the force necessary to maintain a nodule at the surface. However, it is not clear how a horizontally moving bottom current could move a partially buried nodule in a vertical direction without also suspending a quantity of fine sediment, which, on redeposition, would bury the nodules deeply. There are two other arguments against this mechanism.

First, if currents are responsible for rolling nodules over, large nodules are more likely to be buried than small nodules. Heath⁸ has shown, however, that burial of nodules is independent of nodule size. Second, nodules retain the same orientation in the sediment for several 100,000 yr. According to Glasby, development of the different surface textures observed on the top and the bottom of nodules requires $\sim 2.5 \times 10^5$ yr. Radiometric analysis of nodule tops and bottoms^{14,15} also requires that nodules maintained a single orientation for $0.5\text{--}5 \times 10^5$ yr. Polynucleated nodules are frequently recovered from the sea floor. If 0.5 mm of concretion is needed to hold two nodules together, several 100,000 yr are required during which the two nodules could not have moved with respect to each other. Thus nodules are thought to turn over no more often than every 250,000–500,000 yr. The amount of sediment accumulating during this time in most nodule areas is several tens of centimetres, enough to bury nodules deeply. Thus, any dynamic process that turns nodules over cannot maintain them at the surface.

Table 1 Comparison of sediment shear strength at a depth of 7 cm in five box cores* and the load exerted by the largest nodules within each box core.

Dome cores station-box core ⁷	Nodule load (p.s.i.)	Sediment strength (p.s.i.)
47–12	0.012	2.04
50–30	0.013	0.92
49–25	0.016	1.00
50–32	0.017	0.72
57–57	0.010	0.73

* The cores were collected in the equatorial North Pacific at the three Domes sites. See ref. 10 for exact locations and depths.

The possible effect of seismic activity¹³ cannot be discounted, but nodules occur¹⁶ in oceans in some of the World's most seismically quiet areas¹⁷.

The role that organisms might have has been variously discussed. Menard¹⁸ has examined bottom photographs which show organisms partially protruding from under nodule edges. He suggested that these organisms may be pushing nodules up. It has been suggested^{19,24} that organisms may maintain nodules at the surface by rolling them over. This mechanism has the same difficulties as any dynamic process. Epifaunal organisms may remove sediment from nodule tops, possibly as part of their feeding activity²⁰. This activity may keep the nodules free of sediment temporarily but it does not contribute to the down fluxing of sediment beneath nodules. In the absence of sediment down fluxing, the sea floor would soon develop a waffle-like pattern, with a nodule occupying the depression. No such evidence has been found in hundreds of stereophotographs taken of the seafloor in the equatorial North Pacific. Instead, sediment in internodule areas is lower than the nodule tops.

Bottom photographs indicate that organisms actually bury nodules. In many bottom photographs that show a nodule coverage of 25%, areas barren of nodules often correspond to mounds. These mounds, either circular or elongate, in plan view and a few centimetres high, often have a small caldera at the top. Mounds of this type are never surrounded by nodule concentration significantly different from that observed throughout the photograph (Fig. 1a). Clearly these mounds are constructed by infauna that excavate sediment from beneath and pile it on top of the nodules. This mechanism may allow nodules to drift

downward, and become buried. It would also allow nodules to turn, or rotate, during any downward plunge of a few centimetres, provided that undermining was not laterally uniform.

These mounds are superimposed on a sea floor topography consisting of ridges and depressions which have a relief of ~5 cm. These depressions, 15–25 cm across and having a relatively high nodule coverage, are usually surrounded by a ridge which slopes more steeply into than away from the depression. The steeper slope often has a relatively low nodule coverage, suggesting that nodules have drifted down slope into the depression.

How do nodules remain unburied at the sediment surface without reorientation for up to 500,000 yr and how does sediment end up beneath a relatively motionless nodule? The mechanism must transport sediment beneath the nodule at a rate fast enough to overcome sedimentation of new material as well as to overcome the activity of mound-building organisms. Bioturbation might provide the appropriate downward sediment flux which leaves the nodules exposed and undisturbed.

Bioturbation by infauna can transport sediment with two possible effects. (1) Organisms can excavate a volume of sediment at depth and fill in with an equal volume of sediment. The fill material may or may not be more compacted than the original sediment. It will probably contain a fraction of recently deposited surface sediment as this sediment should contain the greatest percentage of labile organic matter. Replacement by more compacted sediment produces a net downward flux but does not lift the sea floor. (2) Organisms can wedge through the subsurface sediment and fill in with some fraction of the uppermost surface material. This option results in lifting of the sea floor, that is, nodules and the surface veneer of sediment. At a depth as great as several millimetres below the nodules, wedging and filling might well leave surface nodules undisturbed.

Bioturbation and downfluxing of sediment through internodule areas are strongly supported by mottling (Fig. 2) and by the frequent occurrence of mixed fauna in the uppermost few tens of centimetres of sediment. Quaternary species decrease in relative abundance with depth in the sediment. Quaternary plankton often are mixed with those of Tertiary age, and the Quaternary species decrease in relative abundance with depth in the sediment²¹. Examination of 25 box cores taken at the three Domes sites supports excavation, however, as opposed to wedging. In three cores where cross-cutting burrows are well preserved (Fig. 2), the older burrow seems to be undeformed by the younger burrow.

For manganese nodules to remain at the surface of the sea floor, sediment must be transported and redistributed beneath them in a way that does not allow the nodule to move with respect to its immediate surroundings. Several tens of centimetres of sediment must be fluxed downward through internodule areas, during which time the orientation of the nodule is not changed. Active mechanisms, which require rotation of the nodules, cannot account for their occurrence at the surface of the sea floor. Wedging through the sediment by infauna such as annelid worms and pelecypods—organisms common to the abyssal environment²²—and infilling with surface material could provide the mechanism. This type of activity within the pelagic environment, may account for the occurrence of ridges and depressions, which have a relief of a few centimetres (Fig. 1b). Wedging by earthworms has been documented by Darwin²³, but this mechanism for the deep ocean is supported more by default of other processes than by observations.

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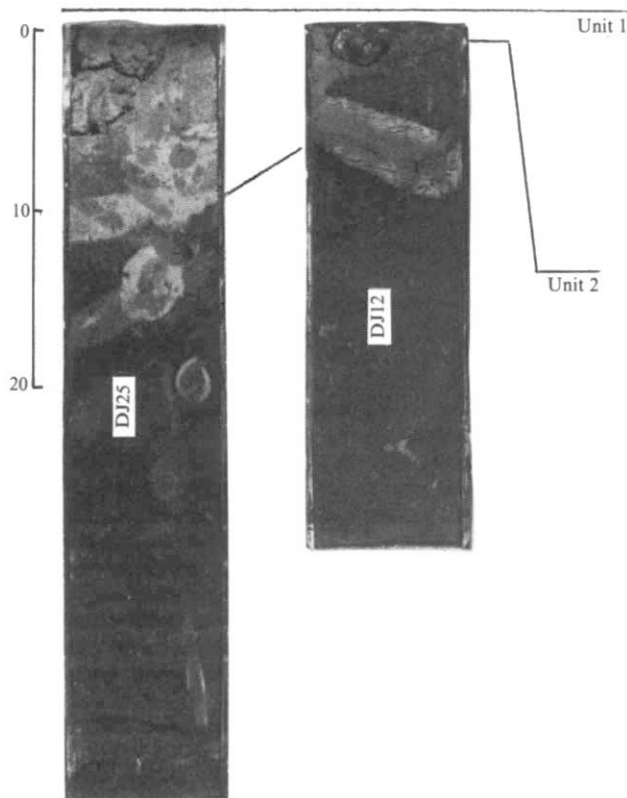


Fig. 2 Subcores from box cores DJ-25 and -12 at Domes Site A, 8°27' N, 150°47' W, ~5,200 m. Intersecting burrows are present in DJ-25 at a depth of 10 cm and 14 cm. The nodule in DJ-12 rests on ~0.2 cm of light-coloured surface sediment, which also fills the large burrow at 6 cm depth. Scale, 20 cm. Unit 1: 0.2–10 cm thick; strongly mottled; greyish-orange and pale yellowish-brown; 95% Quaternary radiolaria; surface nodules have granular texture and 2–10 cm diameter. Unit 2: Greyish-brown mottling with surface sediment; dominantly Miocene–Eocene radiolaria with <1–5% Quaternary radiolaria.

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Fraction dependent variation of aspartic acid racemization age of fossil bone

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The extent of racemization of amino acids provides valuable information on the chronology of various fossil materials¹⁻³. In bone, aspartic acid has received the most attention, and the D/L ratio of this amino acid has been used to estimate ages of human skeletal remains⁴⁻⁹. These estimates were, however, based on the degree of racemization determined for total remnant aspartic acid which may exist in various forms (that is, protein, peptide and free amino acid). The effect of the chemical state of amino acids on amino acid racemization rates or ages in calcareous marine sediments¹⁰ and shells¹¹ has been discussed. In bones, the non-dialysable material in a heated modern sample has a lower racemization rate (isoleucine) than the total organic matrix¹²; free amino acids in fossil bone are more highly racemized than are bound (HCl-insoluble) amino acids¹³. The present investigation was designed to assess such fluctuation of aspartic acid racemization age for a given bone specimen as caused by the variability of the form in which the amino acid is present.

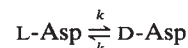
Bones for amino acid analysis were taken from the layers 20, 23B and 25B of the Taishaku Kannondo cave site, Taishaku Ravine, Hiroshima Prefecture in western Japan. All the specimens were long bone fragments of deer or wild boar. A radiocarbon date for a cultural horizon comparable to the layer 20 is $10,085 \pm 320$ yr BP (I-943)^{14,15}. On the basis of stratigraphical and tephrochronological comparisons^{14,16}, we assume that the layer 23B is dated 16,000 yr BP. Layer 25B is of unknown age, but is overlain by the calcic layer of 24A, which was radiocarbon-dated¹⁷ at $20,150 \pm 300$ yr BP. An outline of the archaeological contexts of the Taishaku Kannondo site is given elsewhere^{14,15}.

A small piece (~0.3 g) of transverse section of *Substantia compacta*, cut from each fossil bone, was carefully cleaned by ultrasonication in 0.1 M HCl, ether and double-distilled water. After cleansing, the bones were pulverized and mixed in layers to make an homogeneous powder weighing 1-2 g. Amino acid

fractions were isolated using the procedure outlined in Fig. 1. The ratios of D to L-aspartic acid were determined by gas-liquid chromatography of the *N*-trifluoroacetyl isopropyl esters on a 30 m × 0.28 mm i.d. glass capillary coated with *N,N'*-[2,4-(6-ethoxy-s-triazine)diyl]-bis-(L-valyl-L-valine isopropyl ester)¹⁸ (AE-402, from Gasukuro Kogyo, Inc.).

Figure 2 shows a chromatogram obtained for the Tot fraction in the sample from the layer 23B. Table 1 includes the D/L aspartic acid ratios for Ins, Sp and Tot fractions. Insufficient quantities of amino acids, except glycine, for analysis were available in any Sf fractions, and even the glycine was <0.2% relative to that in Ins fraction. This would be due to removal of free amino acids by percolation of ground water or by the ultrasonic cleansing².

The interconversion of aspartic acid enantiomers is a reversible first order reaction, represented by:



where k is the rate constant. Expressed in the integrated form as a function of time (t), the racemization age equation can be written as

$$\ln \alpha_t - \text{constant} = 2kt$$

where $\alpha = (1 + \text{D-Asp/L-Asp}) / (1 - \text{D-Asp/L-Asp})$ at time t after the start of the reaction. The integration constant is a correction mainly due to the small amount of racemization that takes place during sample preparation procedures. For example, the value of this constant for Tot fraction, calculated from our datum (D/L = 0.063) obtained for a modern bone, is 0.126. However, where it is possible to use two bones from a single site, the dates (t_1, t_2) of which lie at a time interval suitable for 'calibration'⁴, the rate constant of racemization during the interval $t_1 \sim t_2$ is given by:

$$2k = (\ln \alpha_{t_1} - \ln \alpha_{t_2}) / (t_1 - t_2).$$

When $t_1 > t_2$, and t_2 is the age of the start of the Holocene (~11,000 yr), this equation yields the pre-Holocene k value. Thus the k values in Table 1, calculated with the samples from the layers 20 and 23B used for 'calibration', correspond approximately to the pre-Holocene rate constants for the respective fractions. This procedure eliminates the need of setting the enantiomeric ratio for modern bone in the aspartic

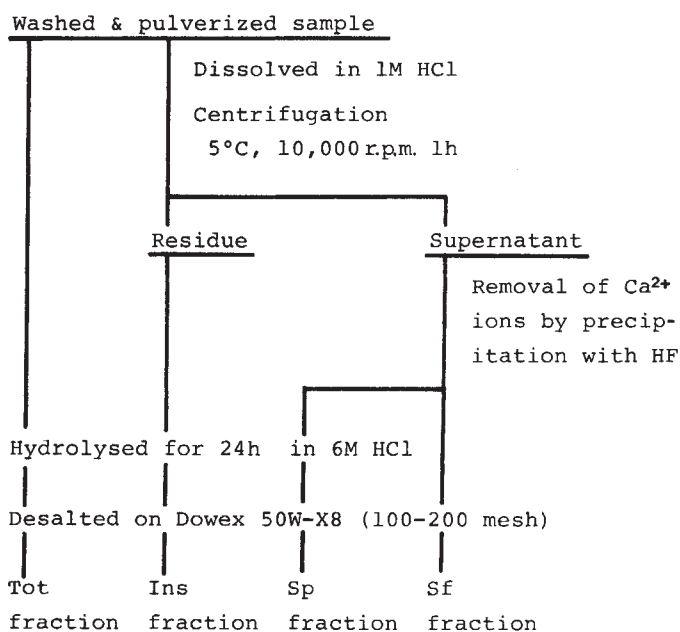


Fig. 1 Fractionation of remnant amino acids in fossil bone. Tot, Total amino acid; Ins, insoluble (protein); Sp, soluble peptide-free amino acid; Sf, free amino acid.

acid racemization age equation, thus minimizing the errors that result from the changes in, for example, concentrations of noncollagen proteins^{19,20} and metal ions in bone with the lapse of time in geological environments. Note that our k values are not directly comparable with the late Pleistocene k values presented by Bada *et al.* (see, for example, ref. 1), as we subtract out the greater part of the Holocene k value.

As Table 1 shows, the order of racemization rates is Sp fraction > Tot fraction > Ins fraction, comparing well with that observed in foraminifera¹⁰ or in shell¹¹. This result is also consistent with what Bada¹² found for the total bone organic fraction versus the non-dialysable fraction.

The racemization age of the layer 25B sample is calculated from

$$t = t_2 + (\ln \alpha_1 - \ln \alpha_2) / 2k.$$

Substituting 10,085 for t_2 , and with the other relevant data, we have 23,700 yr BP (Ins fr.), 19,900 yr BP (Sp fr.) and 22,900 yr BP (Tot fr.). The D/L Asp data in Table 1 give the proportions of aspartic acid in peptide to protein form (Sp/Ins-Asp) in bone. These values are 41, 48 and 63% for the samples from the layers 20, 23B and 25B respectively. The increase in Sp/Ins-Asp value in the longer-buried bones suggests the continuous degradation of collagen. The introduction of the collagen-breakdown product (less racemized) into the Sp fraction may induce the lower age estimate for this fraction. Preliminary analyses of main components of amino acid extracts showed that these Sp fractions are collagenous in composition, although the Hypo/Pro and Ala/Pro ratios in Sp fractions are ~17% and 23% reduced relative to those in respective Ins fractions. The above findings would have a notable bearing on the radiocarbon dating of bone. The HCl-insoluble fraction, which is usually used for ¹⁴C dating, becomes less and less abundant with time. Hence, it would be very interesting to radiocarbon-date the HCl-soluble fraction to which little attention has previously been paid.

The two racemization ages of the Ins and the Tot fractions are compatible with the radiocarbon date of the layer 24A. However, the age estimate based on the total aspartic acid could vary with the proportion of this amino acid in soluble to insoluble state. Variance of Sp/Ins-Asp value in the layer 25B sample, for example, from 50 to 70% should yield the racemization age of 20,000–24,000 yr BP for the Tot fraction.

Such fractional amino acid analyses provide a way of aspartic acid racemization-dating of fossil bone. Where the Sp fraction has a collagenous origin, it is useful for estimating a minimum (or maximum in the case of dating bone with an age less than that of the calibration sample) age, which because of its faster racemization rate is particularly promising for the younger fossils. The aspartic acid racemization reaction for the Ins fraction seems to be a basis to give reliable age estimates for older fossil bones with ages ranging several hundred thousand yr BP. For the

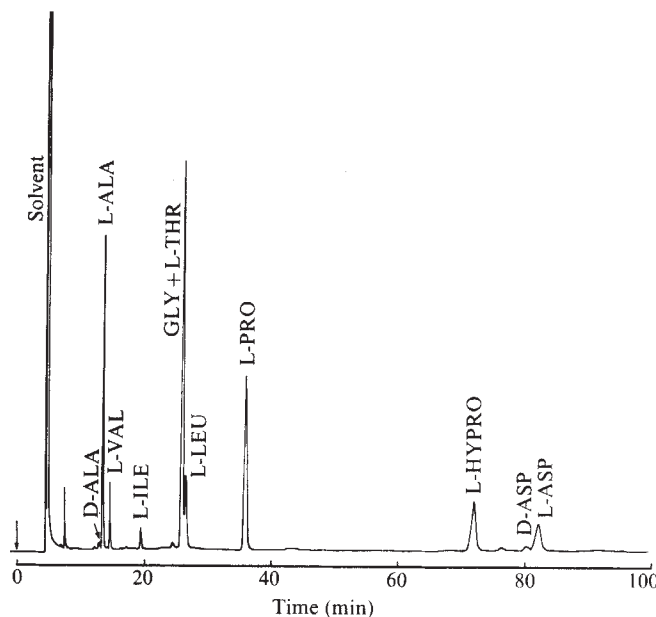


Fig. 2 Gas chromatogram of *N*-trifluoroacetyl isopropyl esters of amino acids recovered from the total hydrolysate of the layer 23B sample from the Kannondo cave site. Chromatography: 30 m × 0.28 mm i.d. column coated with *N,N'*-[2,4-(6-ethoxy-s-triazine)diyl]-bis-(*L*-valyl-*L*-valine isopropyl ester); temperature, 135 °C isothermal; He pressure, 7 p.s.i.; FID detector.

Tot fraction, the degree of amino acid racemization could be dependent on the dynamics of collagen decomposition. Provided that the ratio of partially degraded to pure collagen exhibits a steady increase with burial time (as is supposed to be the case with bones from a single site), a measurement of the extent of racemization on the total hydrolysate would still have archaeological significance. Where the same calibration is applied to fossil samples with different environmental factors involving the disintegration rate of collagen (probably mainly due to humidity in the site), assuming the effective temperature for racemization is nearly identical, then misleading age estimates would be generated.

The present approach would also be useful in overcoming the obstacle caused by the difference in the 'leaching effect'²¹ and in improving the reliability of the racemization dating method.

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Table 1 Aspartic acid racemization in fossil bones from the Kannondo cave site

	Layer	D/L Asp	Aspartic acid age (yr BP)
Ins fraction	20	0.084	$k = 1.70 \times 10^{-6} \text{ yr}^{-1}$ 23,700
	23B	0.094	
	25B	0.107	
Sp fraction	20	0.199	$k = 9.08 \times 10^{-6} \text{ yr}^{-1}$ 19,900
	23B	0.250	
	25B	0.283	
Tot fraction	20	0.115	$k = 4.30 \times 10^{-6} \text{ yr}^{-1}$ 22,900
	23B	0.140	
	25B	0.169	

Analysable amounts of aspartic acid have not been contained in any Sf fractions.

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Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria

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Specific strains of the *Pseudomonas fluorescens-putida* group have recently been used as seed inoculants on crop plants to promote growth and increase yields. These pseudomonads, termed plant growth-promoting rhizobacteria (PGPR), rapidly colonize plant roots of potato, sugar beet and radish, and cause statistically significant yield increases up to 144% in field tests¹⁻⁵. These results prompted us to investigate the mechanism by which plant growth was enhanced. A previous study indicated that PGPR increase plant growth by antagonism to potentially deleterious rhizoplane fungi and bacteria, but the nature of this antagonism was not determined⁶. We now present evidence that PGPR exert their plant growth-promoting activity by depriving native microflora of iron. PGPR produce extracellular siderophores (microbial iron transport agents)⁷ which efficiently complex environmental iron, making it less available to certain native microflora.

Plant growth-promoting fluorescent *Pseudomonas* strains A1, BK1, TL3B1 and B10, isolated from potato periderm or roots, exhibited *in vitro* antibiosis on King's medium B⁸ agar plates (KB) against the bacterium *Erwinia carotovora*, which causes potato soft rot and seedpiece decay (Fig. 1). When KB plates were amended with 1 μM FeCl_3 , the antibiosis against *E. carotovora* did not occur, nor was the yellow-green fluorescence of PGPR produced. The above PGPR also exhibited on KB plates *in vitro* antibiosis against *Escherichia coli* K-12 AN193, which does not produce its native siderophore, enterobactin, but not against its enterobactin-producing parent, *E. coli* K-12 AN194. These results suggested that in iron-deficient conditions, PGPR were producing fluorescent siderophores which were responsible for antibiosis against *E. carotovora* and *E. coli* K-12 AN193. Yellow-green fluorescent siderophores have previously been isolated from iron-limiting cultures of *P. fluorescens*⁹⁻¹¹.

We next investigated the effect of iron on plant growth-promoting activity by PGPR in soils. When potato seedpieces were dipped into PGPR suspensions (10^9 Colony-forming units (CFU) per ml) immediately before planting in field soils in a previously described greenhouse assay⁵, statistically significant increases in plant growth (total plant wet weight) occurred 2 weeks after emergence. Average increases (six replications with three plants each) were 47% for strain A1, 63% for strain B10 and 74% for strain BK1. The control was an autoclaved suspension of strain A1. In contrast, when 120 ml of 100 μM ethylenediaminetetra-acetateferrate (III) (Fe^{III} EDTA⁻) per 600 g of soil were added on alternate days to soils planted with PGPR-inoculated potato seedpieces, PGPR did not statistically increase growth ($\pm 4\%$). Addition of iron alone to soils did not significantly increase plant growth over the control. The effect of iron (III) on colonization of roots by PGPR was examined with strain A1 as previously described^{2,6}. Although the addition of Fe^{III} EDTA⁻ resulted in no increase in plant growth, the bacteria, nevertheless, colonized roots at populations averaging 3.2×10^3 CFU per cm root as compared with 4.4×10^3 CFU per cm without iron.

These results suggested the intriguing proposition that a fluorescent siderophore from PGPR may mimic the biological action of PGPR. To test this, we isolated a yellow-green fluorescent pigment capable of binding iron (III) from broth cultures of strain B10 grown in an iron-deficient medium containing glycerol and proteose peptone no. 3. The pigment was purified to homogeneity by a procedure similar to that of Meyer and Abdallah¹⁰. This pigment exhibited properties typical of a siderophore, including complete repression of production in culture media containing micromolar amounts of iron (III). Furthermore, both the pigment and its ferric complex reversed iron starvation of strain B10 on KB plates induced by the synthetic ferric complexing agent, ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA), which is not utilized by the cells¹². As the chemical and physical properties of this siderophore seem to be different (J.L. and M.T., unpublished results) from those of others isolated earlier^{10,11,13-15}, we name this new siderophore, pseudobactin. When paper disks containing 20 μl of 200 μM pseudobactin were applied to KB plates previously seeded with *E. carotovora*, 3-mm radial growth inhibition zones about the disks were observed within 12–16 h. Red-brown ferric pseudobactin caused no antibiosis against *E. carotovora* up to millimolar concentrations. These results suggest that the *in vitro* antibiosis of PGPR against *E. carotovora* is caused by iron deprivation induced by the PGPR's siderophore. *E. coli* K-12 AN194 was not susceptible to PGPR because enterobactin may be able to reverse iron starvation precipitated by PGPR's siderophore.

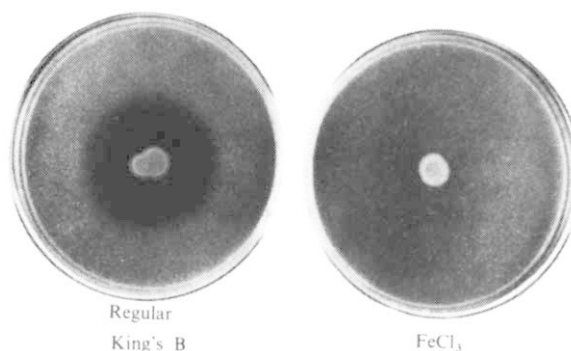


Fig. 1 Plant growth-promoting rhizobacterial antibiosis against *Erwinia carotovora* eliminated by iron (III). Both King's B agar plates were inoculated in the centre with 10 μl of a suspension of fluorescent *Pseudomonas* strain B10 ($\sim 10^6$ CFU ml^{-1}), incubated for 24 h at 28 °C, and sprayed with a suspension of *E. carotovora* ($\sim 10^6$ CFU ml^{-1}). The plate on the right contained 10 μM FeCl_3 , whereas the plate on the left contained no added iron (III). The zone of growth inhibition of *E. carotovora* and the fluorescence of strain B10 were examined at 24 h. Similar results were obtained with fluorescent *Pseudomonas* strains A1, BK1 and TL3B1.

The effect of pseudobactin on plant growth and rhizoplane fungal colonization was determined in the greenhouse assay (Table 1). Both B10 suspensions and pseudobactin at 10 μM caused significant plant growth increases compared with water-treated controls. Ferric pseudobactin at 50 μM and B10 with 50 μM Fe^{III} EDTA⁻ did not increase plant growth. In addition to increasing plant growth, pseudobactin and B10 treatments resulted in statistically significant reductions of fungal populations on the rhizoplane relative to the water-treated control. Fungal colonization of the rhizoplane, measured by the method of Huisman *et al.*¹⁶, averaged 5.5 CFU per 10 cm root on water-treated plants compared with 2.3 CFU per 10 cm (-59%) on B10-treated plants and 1.4 CFU per 10 cm (-74%) on pseudobactin-treated plants.

Based on these results, we propose the following scenario to account for the enhancement of plant growth by PGPR.

Following inoculation and planting of crop seeds, PGPR rapidly colonize roots of the developing plant. As a result of a limited supply of iron in the rhizoplane, PGPR produce siderophores which sequester iron in the root zone, making it unavailable to certain rhizoplane microorganisms. These microorganisms are unable to obtain essential quantities of iron for growth either because they do not produce siderophores, produce comparatively less siderophores than those of PGPR, and/or produce siderophores which have less affinity for iron than those of PGPR. The populations of these microorganisms, some of which are quasi-pathogens (T. V. Suslow and M.N.S., unpublished

Table 1 Enhanced plant growth of potato by pseudobactin in greenhouse assay

Treatment	Average plant weight (g)
H ₂ O control	1.0
50 μ M Fe ^{III} EDTA ⁻ control	0.9
B10	2.3*
B10 + 50 μ M Fe ^{III} EDTA ⁻	0.9
10 μ M pseudobactin	2.5*
50 μ M ferric pseudobactin control	1.1

Potato seedpieces were planted in field soil (sandy loam) from Moss Landing, California. In treatments with fluorescent *Pseudomonas* strain B10, seedpieces were dipped in approximately 10^9 CFU ml⁻¹ suspension before planting. Pots were watered with 120 ml of the appropriate solution on alternate days, and a total of nine applications were made. Plants were harvested 10 days after emergence. An average of six replications with three plants per replication were obtained.

* Statistically significant increase ($P = 0.01$) compared with controls.

results), are reduced, and a more favourable environment for root growth is created. Our results with mutants of PGPR support this hypothesis. Certain mutants obtained by treatment with *N*-methyl-*N'*-nitro-nitrosoguanidine or UV light at 254 nm were non-fluorescent, did not exhibit antibiosis against *E. carotovora* and did not promote growth increase of potato in the greenhouse assay even though they were able to colonize plant roots⁶. At least five mutants derived from strains BK1, TL3B1 and TL3B2 were examined. These mutants did not produce siderophores, in contrast to their parents. An alternative hypothesis that plant growth-promoting rhizobacterial siderophores bind iron and make it directly and perhaps more readily available to the plant seems unlikely because ferric pseudobactin did not promote plant growth (Table 1).

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Antiquity of the vertebrate pattern of activity metabolism and its possible relation to vertebrate origins

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Vertebrates generally possess well developed capacities for anaerobic metabolism, resulting in formation of lactic acid. Those capacities have traditionally been interpreted in terms of adaptation to hypoxic environments or to special situations such as diving. However, anaerobic metabolism in striated muscle tissue is frequently a major source of ATP utilized during periods of intense activity. The evolutionary significance of anaerobically supported activity has not been discussed, although the interrelationships of capacities for aerobiosis and activity have received considerable attention¹. We present here evidence that the pattern of activity metabolism utilized by extant species probably dates back to the earliest vertebrates. It is also postulated that the evolution of extensive capacity for anaerobically supported burst activity may have been closely related to the evolution of vertebrates from invertebrate chordates.

Many animals use glycolysis, the anaerobic process by which glucose is degraded to lactic acid. Most invertebrate species resort to significant utilization of glycolysis only in the absence of sufficient environmental oxygen to maintain adequate rates of ATP generation via aerobiosis, or oxidative phosphorylation². However, many vertebrate species rely on prodigious rates of glycolysis for ATP generation during periods of maximal activity^{3–6}. Such a pattern of activity metabolism enables many vertebrates to reach 'burst' levels of activity otherwise unattainable if they were solely dependent on aerobic metabolism. Heavy reliance on anaerobic metabolism also has its drawbacks: it is inefficient in utilizing substrate, and is invariably associated with muscle fatigue⁷. In addition, resultant intramuscular lactic acid accumulation and subsequent diffusion of lactate into the cardiovascular system⁸ disrupts maintenance of blood and tissue pH which may well affect enzymatic activity, protein configuration and so on⁸. This exercise-related pH depression may persist in lower vertebrates for several hours, or longer, following cessation of strenuous activity⁹.

Although some crustaceans generate moderate quantities of lactate during exercise^{10,11}, most invertebrates produce little, if any, lactate during maximal activity. For example, during periods of intense activity, the insects are highly dependent on aerobiosis¹² and molluscs anaerobically generate octapene and succinate to supply themselves with sufficient ATP¹³. Hence, the vertebrate capacity for lactate generation during activity should not be considered an adaptation for anaerobiosis *per se*: it is not merely a primitive relict of the occupation of oxygen-depleted environments, but is a specific and relatively unique exploitation of glycolysis to support intense activity.

Lactic acid formation during activity has been demonstrated in many extant gnathostomes (sharks¹⁴, teleost fish⁶, amphibians³, reptiles, mammals⁵). Those data indicate that heavy reliance on lactate formation during activity is widespread among vertebrates. However, the antiquity of this pattern of activity support is still unclear; is it a primitive characteristic dating back to, or even previous to, the origin of vertebrates? To

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Table 1 Whole body lactic acid concentration and blood pH before and after activity in two species of cyclostomes and whole body lactic acid concentration before and after activity in amphioxus

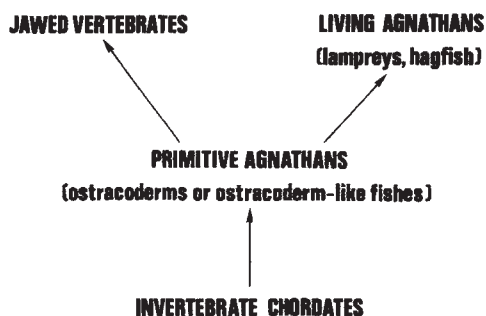
Species	Lactic acid concentration (mg per g tissue)		Blood pH			
	Rest	Active	Rest	30 min post activity	60 min post activity	180 min post activity
Pacific hagfish (<i>Eptatretus stouti</i>)	0.14 ± 0.01 (n = 4)	1.51 ± 0.14 (n = 4)	8.03 ± 0.03 (n = 5)	7.60 ± 0.12 (n = 5)	7.48 ± 0.14 (n = 5)	7.31 ± 0.19 (n = 5)
Pacific brook lamprey (<i>Lampetra pacifica</i>)	0.17 ± 0.01 (n = 5)	1.65 ± 0.18 (n = 7)	7.91 ± 0.04 (n = 5)	7.16 ± 0.06 (n = 5)	—	—
Amphioxus (<i>Branchiostoma caribaeum</i>)	0.14 ± 0.03 (n = 4)	0.29 ± 0.04 (n = 8)	—	—	—	—

Post-active depression of blood pH probably results from protracted diffusion of lactic acid into the cardiovascular system from striated musculature as well as slow subsequent removal of lactate from the circulatory system. Adult *Lampetra* (36) were obtained by dip net in Oak Creek, Benton County, Oregon, in May 1979. Adult *Eptatretus* (28) were caught in 'fike' traps at a depth of 150 m, 1,000 m off the coast of Coos Bay, Oregon in December 1978. Adult *Branchiostoma* (12) were collected in Tampa Bay, Florida, in December 1979. Animals were maintained in 160-l capacity tanks, situated in thermally controlled rooms set at 12 °C on an 11-h photoperiod. Tank water was kept fully aerated at all times. Experiments were performed 4–6 days after animals were captured. Twelve *Lampetra* (mean mass 6.6 g), 9 *Eptatretus* (mean mass 52.2 g) and 12 *Branchiostoma* (mean mass 0.14 g) were used to determine whole body lactate content either at rest or immediately after maximal activity. Animals were placed individually in a 1 m × 1 m × 0.25 m tank of water equilibrated to 12 °C. They were then stimulated to activity by persistent, noxious but harmless prodding. Reaction of all animals to stimulus was intense, and was assumed to represent maximal activity levels. During stimulation, both *Lampetra* and *Eptatretus* underwent an initial period of vigorous activity lasting 2.5–3.5 min. In both species, this consisted of frantic lateral undulations interspersed with frequent attempts to 'bite' the probe. During the following 1.5–2.5 min, animals appeared to tire but continued to exhibit similar types of behaviour. In addition, mucus was secreted from the slime glands of *Eptatretus* during the entire exercise period. This was removed from the tank approximately every 30 s in such a manner that movements by animals were not hindered. During the initial 10–15 s of the exercise period, *Branchiostoma* underwent intense lateral undulation. Thereafter, they appeared to tire significantly and remained essentially refractory to further stimulus. After 5 min of stimulation, animals were decapitated, homogenized in 0.6 M perchloric acid for 2 min in an Oster blender and total body lactate was measured spectrophotometrically by the method of Bennett and Licht⁴. Similar analyses were also made on unstimulated animals to determine lactate levels for resting individuals. Ten *Lampetra* and 20 *Eptatretus* were used to determine the effect of this activity on blood pH. Blood samples of 75 µl were taken in heparinized syringes from five previously undisturbed individuals of each species. Samples were withdrawn from tail incisions in *Lampetra* and from the caudal sinus in *Eptatretus*. Elapsed time between first handling and sample procurement was less than 15 s; struggling by animals was minimal during this time period. The pH of the sample was measured immediately with a Radiometer Copenhagen BMS3Mk2 blood micro unit connected to a Radiometer Copenhagen PHM 71 Mk 2 acid-base analyser. The temperature of the electrode was regulated at 12 °C ± 0.5 °C. This measurement was assumed to represent the pH of the animals' blood at rest. Different individuals of each species were then stimulated to activity for 5 min as described in the previous experiments. Blood samples were collected and analysed (as described for resting individuals) 30 min after cessation of activity in both species. Additional samples were taken 60 and 180 min after activity in *Eptatretus*.

elucidate this question, we investigated patterns of activity metabolism in some living agnathans (Cyclostomata) and the protochordate, amphioxus (Cephalochordata). Morphological evidence indicates long evolutionary isolation of cyclostome and gnathostome lineages as well as independent derivation of both groups from the earliest vertebrates, the ostracoderms of the early Palaeozoic (Fig. 1)¹⁵. Thus, parsimony dictates that occurrence of a particular trait (burst activity supported by anaerobiosis) in both cyclostomes and gnathostomes might be the result of retention of a primitive characteristic present in the common ancestor of both groups.

Activity physiology in extant protochordates, especially cephalochordates, may well be reflective of activity metabolism in the chordate ancestors of vertebrates. Cephalochordates are thought to resemble closely protovertebrates in many aspects of their morphology and in their sedentary, microphagous filter-feeding lifestyle¹⁶. Consequently, their activity physiology may also be similar.

Cyclostomes investigated in this study included the Pacific brook lamprey (Petromyzontidae: *Lampetra pacifica*) and the Pacific hagfish (Myxiniidae: *Eptatretus stouti*). The Pacific brook lamprey is a small, non-parasitic lamprey inhabiting streams west of the Cascade Mountains of the north-west US. These are strictly freshwater forms that never migrate to marine environments. The Pacific hagfish occurs off the west coast of North America from southern California to south-east Alaska in silty-bottom environments at depths of 18–940 m.

**Fig. 1** Phylogenetic relationships of the vertebrates (based on refs 15, 19).

The cephalochordate investigated here was *Branchiostoma caribaeum* (Branchiostomidae). This is a small, burrowing, filter-feeding inhabitant of sandy-bottomed marine environments from Chesapeake Bay to the West Indies.

Both cyclostomes utilized glycolysis extensively during the exercise period (Table 1): lactate concentrations were approximately 10 times greater after 5 min of activity than at rest ($P < 0.05$, t -test). In addition, both species experienced a concomitant, protracted decline in all post-active blood pH measurements ($P < 0.05$, t -test) (Table 1). Quantities of lactate formed as a result of activity and the accompanying extended post-active decline of blood pH in these species closely parallel previously described modes of activity physiology among gnathostomes^{7,9}.

Consequently, utilization of the pattern of activity metabolism described here must be of considerable antiquity, perhaps as old as vertebrates themselves. Figure 1 illustrates the generally accepted phylogenetic relationships of the vertebrates. If these relationships are correct, we must assume the pattern of activity metabolism exhibited by living vertebrates is a result of either (1) convergent evolution between cyclostome and gnathostome lineages; or (2) retention of a primitive vertebrate characteristic. Insofar as premise (2) is the most parsimonious, it must be considered the most likely to describe the actual sequence of events.

The implications of that conclusion for the evolution of vertebrates are manifold. For example, parameters of anaerobically supported activity (such as capacity for high levels of burst activity, fatigue, extended post-active blood and tissue pH alteration) have probably vitally affected adaptive radiation of the morphology, physiology and behaviour of virtually all the major groups of vertebrates. In fact, only with the evolution of homeothermy in birds and mammals have aerobic potentials been expanded to the point where, for example, they can support very high levels of activity without consequent fatigue¹⁷.

In contrast, the chordate ancestors of vertebrates may not have possessed such well developed capacities for burst activity as early vertebrates. The cephalochordate studied here was incapable of any more than 10–15 s of intense lateral undulation and generated only minimal quantities of lactate during exercise (Table 1). This pattern of activity physiology is hardly surprising:

cephalochordates, like all adult invertebrate chordates, lead a sedentary, filter-feeding existence and seldom, if ever, take any more than the briefest of forays into the water column. Thus, if current interpretations regarding vertebrate origins are correct (see above), one might reasonably postulate a similarly minimal metabolic capacity for intense activity in the chordate ancestors of vertebrates.

Those differences in capacity for activity between invertebrate chordates and the earliest vertebrates might indicate concomitant and interdependent evolution of vertebrate activity physiology and the appearance of the vertebrates themselves. Halstead¹⁸ and others have rightly emphasized that the earliest vertebrates lacked a vertebral column, but differed from invertebrate chordates in that the latter group never exhibits a high degree of cephalization. Accordingly, as described above,

all adult invertebrate chordates are at least relatively sedentary, whereas vertebrates are among the most active of the Metazoa. Thus, vertebrate cephalization may have developed in response to selection for increased sensory and locomotor control as a more active lifestyle evolved. We suggest that the expanded vertebrate capacity for burst activity contributed significantly to that active mode of existence. Consequently, evolution of the vertebrate pattern of activity metabolism may well have been a selective factor contributing to cephalization in protovertebrates and the appearance of vertebrates themselves.

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Human cell-surface glycoprotein with unusual properties

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We report here the identification of human cell-surface glycoprotein with unusual and interesting properties. We initially detected this glycoprotein on the surface of cultured human haematopoietic cell lines by means of a monoclonal antibody. Although it is expressed on all cultured human haematopoietic cell lines tested, including the inducible promyelocytic tumour cell line HL-60 (ref. 1), it is not present in readily detectable amounts on most normal or leukaemic human haematopoietic cells. HL-60 cells, on exposure *in vitro* to appropriate chemical inducers, undergo morphological and functional differentiation along the granulocytic pathway or into macrophage-like cells²⁻⁶. One consequence of *in vitro* induction is the specific loss of the glycoprotein from the surface of HL-60 cells. The molecule does not, however, seem to be a highly tissue-specific differentiation antigen because it is also found on human tumour cell lines derived from non-haematopoietic tissues. Rather, its expression seems to be related to cell proliferation. Preliminary chemical characterization suggests that the glycoprotein may be identical to the abnormal glycoprotein previously reported by Bramwell and Harris to be associated with malignancy^{7,8}.

The IgG monoclonal antibody used in these studies is designated B3/25. It was obtained from fusion of BALB/c mouse spleen cells immunized against the human haematopoietic cell line K562 (ref. 9), with the non-producer myeloma cell line S194/5.XX0.BU.1, by standard procedures^{10,11}.

Trace antibody binding assays¹² showed that B3/25 antibody reacted with 7/7 cultured human T-leukaemic cell lines, 11/11 B-cell lines including 'normal' B-cell lines, B-cell tumours and Burkitt's lymphoma cell lines, and 10/10 non-T-, non-B-leukaemic cell lines. The antibody precipitated a labelled molecule

from NP40 lysates of iodinated HL-60 cells which migrated with an apparent molecular weight of about 110,000 on SDS-polyacrylamide gels in reducing conditions (Fig. 1). Similar results were obtained in immunoprecipitation studies with other haematopoietic cell lines. This molecule, which on the basis of metabolic labelling studies and lectin affinity chromatography is a glycoprotein, seems to exist in the membrane as a disulphide-bonded dimer, as in nonreducing conditions it migrates in SDS-polyacrylamide gels with an apparent molecular weight of 200,000 (Fig. 1).

Although the glycoprotein was detected on the surface of all the human haematopoietic cell lines tested, we could not detect it on peripheral blood mononuclear cells, granulocytes or a variety of peripheral blood leukaemic cells either by fluorescence-activated cell analysis or by trace antibody binding. A more extensive survey of the distribution of the glycoprotein on normal and leukaemic haematopoietic cells was carried out by immunofluorescence microscopy. The molecule was not present in detectable amounts on a significant fraction of thymocytes, bone marrow or peripheral blood mononuclear cells, nor on leukaemic cells from patients with chronic myeloid leukaemia or chronic lymphatic leukaemia, except for apparently specific staining of a small population of cells morphologically resembling erythroblasts. The majority of blast cells from patients with acute myeloid or lymphocytic leukaemia were also negative. In some cases, however, a small fraction (0-30%) of the blast cell population was positive. In two exceptional cases, one a T-cell acute lymphocytic leukaemia, the other an erythroleukaemia, almost all the blast cells reacted strongly with B3/25 antibody.

In addition to its unusual cellular distribution, we found that the glycoprotein defined by B3/25 monoclonal antibody had another interesting property. Although the molecule is expressed on non-induced HL-60 cells, we detected a dramatic decrease in the amount of glycoprotein on cells induced to differentiate by growth in the presence of 1.25% dimethyl sulphoxide (DMSO) or 0.5 mM sodium butyrate for 5 days (Fig. 2 and ref. 1). The fact that the radioactivity bound to induced HL-60 cells incubated with B3/25 antibody in the trace antibody binding assay is barely above background indicates that the induced cells express little or no antigen. Two other low molecular weight compounds, *N*-methyl acetamide and phorbol ester, that also induce morphological and functional differentiation of HL-60 cells²⁻⁶, cause a similar decrease in the expression of the glycoprotein on HL-60 cells.

We studied the expression of B3/25 glycoprotein on HL-60 cells in more detail by quantitative immunofluorescence using the fluorescence-activated cell analyser. The amount of the glycoprotein found on non-induced cells varied considerably (Fig. 3a), and this heterogeneity was not abolished by recloning the cell line. Approximately 70% of non-induced HL-60 cells expressed sufficient antigen to be detected above nonspecific background fluorescence. After treatment with DMSO for 5 days, only 5% of the cells were scored as positive and the fluorescence profile of the specifically stained cells was indistinguishable from that of the control (Fig. 3b). Kinetic studies showed that the loss of B3/25 glycoprotein was detectable within 24 h and essentially complete after 48 h (Fig. 3d). The disappearance of the glycoprotein precedes the arrest of HL-60 cells in G1 of the cell cycle and morphological differentiation, which is not maximal until at least 5 days after induction (Fig. 3d and refs 2, 13). Pulse-chase metabolic labelling experiments show that the loss of the glycoprotein is due entirely to the almost complete cessation of synthesis of the molecule and not to an increased rate of degradation (M.B.O. and I.S.T., unpublished results). As shown in Fig. 3c, we have obtained a variant subline of HL-60 cells that continues to grow in the presence of 1.25% DMSO without undergoing terminal differentiation. In these conditions the cells express similar amounts of B3/25 glycoprotein to non-induced wild-type cells. Thus, the change in expression of the glycoprotein seems to be directly associated with the arrest of growth and differentiation of HL-60 cells *per se*.

Although the cell-surface glycoprotein is not a cell cycle-dependent antigen in a conventional sense, as its expression on

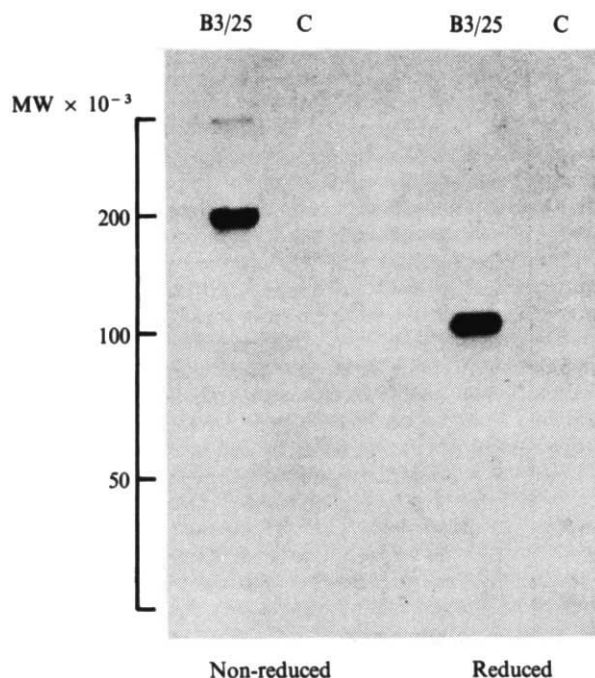


Fig. 1 SDS-polyacrylamide gel analysis of the cell-surface antigen recognized by B3/25 monoclonal antibody. HL-60 cells originally from Dr R. Gallo and recloned at the Salk Institute were iodinated by the glucose-oxidase modification of the lactoperoxidase technique as previously described¹⁸. Immunoprecipitates were prepared from cell lysates containing 1% NP40, 1% deoxycholate and 0.1% SDS using B3/25 antibody and adsorbing the immune complexes to *Staphylococcus aureus* essentially as described previously¹⁹ except that goat anti-mouse IgG was used as the second antibody. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis in 7.5% gels²⁰ after reduction with 2-mercaptoethanol or treatment with 100 mM iodoacetamide. Control immunoprecipitates from which B3/25 antibody was omitted were also prepared and analysed (gel tracks marked C). The autoradiograph shown was exposed for 1 h with the use of an intensifying screen²¹ to enhance autoradiography. MW, Molecular weight.

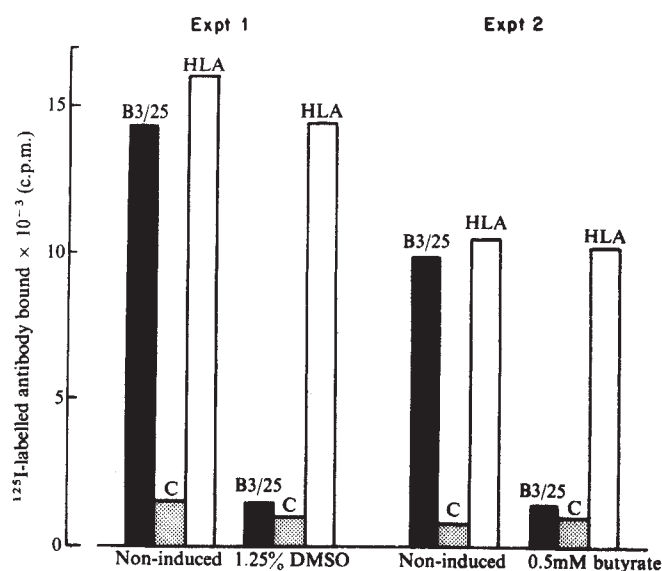
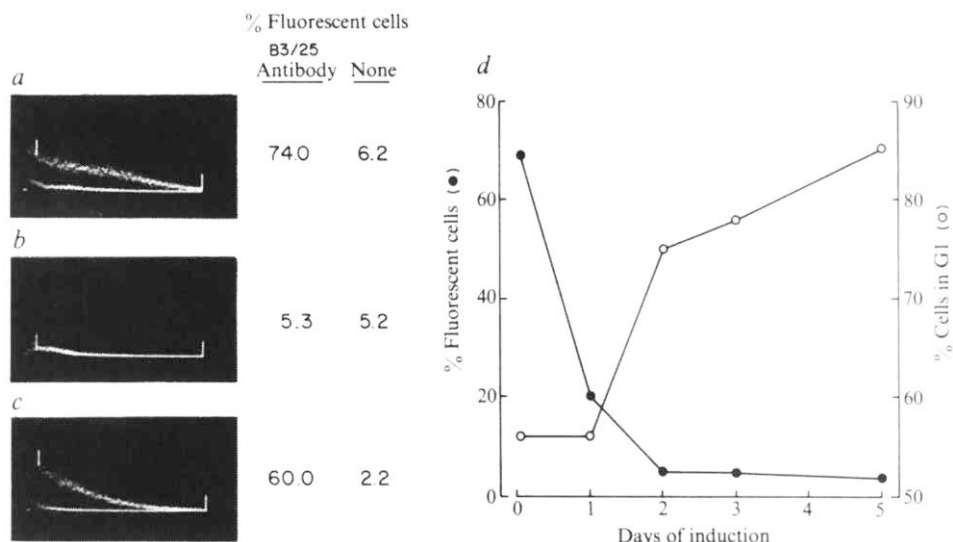


Fig. 2 Comparison of the binding of B3/25 antibody to induced and non-induced HL-60 cells. HL-60 were routinely maintained in RPM1 1640 medium supplemented with 10% fetal calf serum and 10 μ M 2-mercaptoethanol. For induction experiments, cells were collected and resuspended at 2×10^5 cells per ml in fresh culture medium alone or containing either 1.25% (v/v) DMSO or 0.5 mM Na butyrate. After growth for 5 days, the cultures were collected and binding of B3/25 monoclonal antibody to non-induced and induced HL-60 cells determined by a trace antibody binding assay using 125 I-labelled affinity-purified rabbit F(ab')₂ anti-mouse IgG antibody essentially as described by Morris and Williams¹². In the negative control for nonspecific binding (column C), tissue culture medium was substituted for culture supernatant containing B3/25 antibody. Binding of a monoclonal antibody designated A3/10 which reacts with a common antigenic determinant on HLA molecules (M.B.O. and I.S.T., unpublished results) was used as a positive control. In expt 1, 5×10^5 cells were used per assay, in expt 2, 2×10^5 cells per assay were used and in both cases the results shown represent the average of duplicate determinations.

induced HL-60 cells changes before the cells arrest in G1 of the cell cycle, all the previous results are consistent with the molecule being selectively expressed on actively proliferating cells. We therefore examined whether or not the molecule was present on blast cells derived from lectin-stimulated peripheral blood lymphocytes. Trace antibody binding and quantitative immunofluorescence suggested that the glycoprotein was present on the blast cells, but in such small amounts relative to the cultured cell lines that we considered the results equivocal. However, the presence on lectin-induced blasts of a similar or identical glycoprotein to that found on cell lines could be clearly demonstrated by immunoprecipitation from lysates of iodinated cells (Fig. 4). Thus, the glycoprotein or a related molecule is clearly present on normal proliferating cells. There may, however, be quantitative differences between the expression of the glycoprotein on tumour cells and normal cells and there is no evidence that the glycoprotein on the blast cells is identical to the molecule found on the tumour cell lines. These considerations are particularly relevant because, although there has been no previous report of the presence on human haematopoietic cells of a glycoprotein with similar properties to those of the surface glycoprotein defined by B3/25 monoclonal antibody, it is probable that the molecule we are studying is related to a tumour cell-surface glycoprotein described earlier by Bramwell and Harris^{7,8}. This glycoprotein seems to be expressed in greater amounts on malignant cells than on nonmalignant cells and may also be structurally modified in tumour cells.

The most compelling evidence that the two glycoproteins are closely related is that both have the same apparent molecular weight on SDS-polyacrylamide gels and share the unusual

Fig. 3 Expression of B3/25 glycoprotein on induced and non-induced HL-60 cells measured by quantitative immunofluorescence. Cells were grown in the presence of 1.25% (v/v) DMSO for the period of time indicated and then stained with saturating amounts of B3/25 antibody and a fluorescein-conjugated affinity-purified goat anti-mouse IgG. Cells (50,000) were analysed on a Los Alamos-type fluorescence-activated cell analyser using a laser output of 900 mW, high voltage setting of 800 V, and a fluorescence gain of 1.0. Panels *a-c* show the histograms of non-induced HL-60 cells (*a*), HL-60 cells induced with 1.25% DMSO for 5 days (*b*), and a DMSO-resistant variant of HL-60 grown in 1.25% DMSO for 5 months (*c*) after staining with B3/25 antibody. Also shown are controls for nonspecific staining in which B3/25 antibody was omitted from the first incubation. The % of fluorescent cells within the channels indicated by the vertical bars are given. *d* Shows the % of B3/25 positive cells determined by flow microfluorimetry in the conditions just described after exposure of HL-60 cells to DMSO for various periods of time. The cell-cycle distribution of the same cells was determined from their DNA content measured by flow microfluorimetry after staining with mithramycin²². The % of cells in G1 is indicated.



property of existing in their native states as disulphide-bonded dimers. The glycoprotein we have studied is also similar to the molecule identified by Bramwell and Harris in other respects: it is acidic, binds to concanavalin A and wheat-germ agglutinin, and is expressed on Hela cells and other non-haematopoietic tumour cell lines (M.B.O. and I.S.T., unpublished results). Furthermore, by quantitative absorption we could not detect the glycoprotein on any normal tissue tested. Homogenates of liver, brain and kidney and red blood cells were used in the absorptions and the limit of detection was about 5% of the amount expressed on RPM1 8402, the human T-leukaemic cell line used as a positive control in these experiments.

Regardless of whether, as seems likely, the glycoprotein we have characterized is related to the one described by Bramwell and Harris, its properties warrant further investigation. Quantitative studies of the expression of the glycoprotein on normal tissues and *in vivo* tumours are required, as well as chemical characterization of the molecule isolated from both non-malignant and malignant cells. It may be possible to determine whether or not there is a causal relationship between the expression of the glycoprotein and cell proliferation by means of somatic cell genetics using HL-60 cells as a model system. It will also clearly be important to establish whether the glycoprotein is expressed on normal or leukaemic haematopoietic stem cells that can be detected by the appropriate *in vitro* assays¹⁴⁻¹⁷. The B3/25 monoclonal antibody and other monoclonal antibodies which we have obtained that react with different antigenic determinants of the same glycoprotein (I.S.T., unpublished results) will facilitate these studies.

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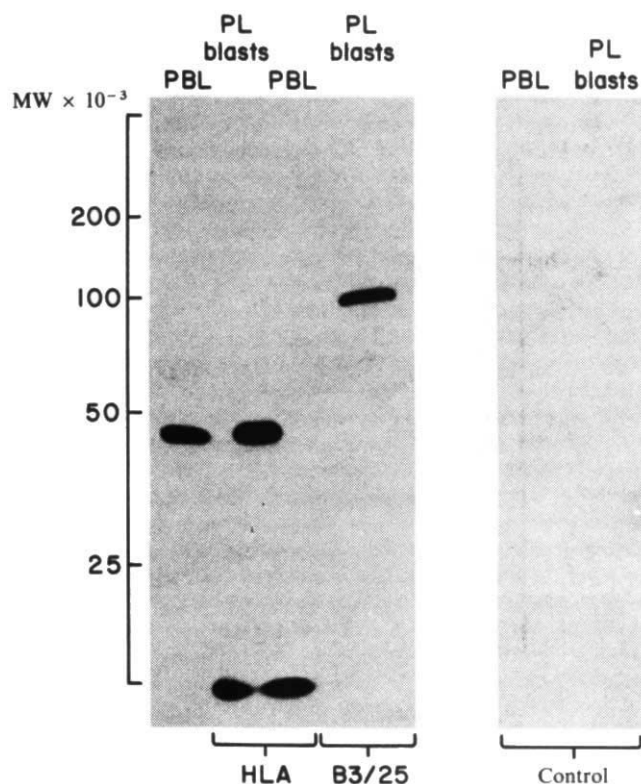


Fig. 4 Expression of B3/25 glycoprotein on lectin-stimulated peripheral blood lymphocytes (PBL). Mononuclear cells were isolated from defibrinated blood by centrifugation of Ficoll-Hypaque gradients. Fresh cells (PBL) or cells (2×10^6 per ml) stimulated for 5 days with $20 \mu\text{g ml}^{-1}$ pea lectin²³ in RPM1 1640 medium supplemented with 10% fetal calf serum (PL blasts) were iodinated and immunoprecipitates prepared and analysed as described in Fig. 1 legend except that a 10% polyacrylamide gel was used. A3/10 antibody was used to precipitate HLA as a positive control. A negative control in which B3/25 antibody was omitted from the first incubation is also shown. Exposure of the autoradiograph was 40 h.

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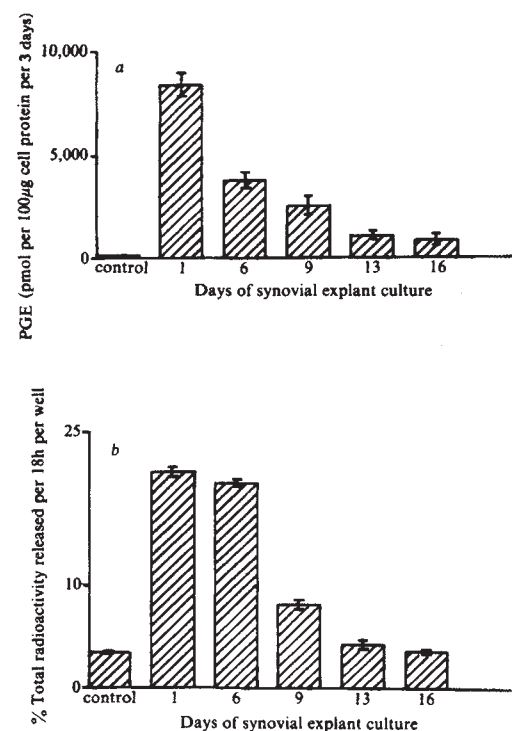


Fig. 1 *a*, Stimulation by SEM of PGE production by chondrocytes. The results represent the mean $\pm$ standard error of determinations in quadruplicate wells. A dilution of 1:25 (final concentration 4% v/v) of conditioned SEM in fresh MEM containing 10% FCS was added to each well containing confluent cultures of chondrocytes (second passage), derived from explants of human articular cartilage obtained from amputation specimens. Control cultures contained no SEM. Values have been corrected by subtracting the PGE present in the synovial medium (22.0%–59.0% of total PGE measured in each well) and therefore represent the PGE produced by the chondrocytes themselves. *b*, Stimulation by SEM of production of plasminogen activator by human articular chondrocytes. Results expressed as mean $\pm$ standard error ($n = 3$ wells). Chondrocytes were grown to confluence in MEM containing 10% FCS and 20 mM HEPES buffer with 0.085% (w/v) NaHCO_3 . Then the FCS was replaced by 10% (v/v) acid-treated FCS (acid treatment destroys the proteinase inhibitor, α_2 -macroglobulin) and 4% (v/v) SEM was included in the medium. Controls did not contain SEM. Culture medium was removed after 18 h and counted for liberated ^{125}I . Residual fibrin was digested from the wells with 0.1% trypsin and this was also counted; fibrin degradation by chondrocytes was then calculated as a percentage of the total present. No significant release of ^{125}I was apparent during the preincubation of chondrocytes in normal FCS. Additional control wells containing SEM but no chondrocytes showed no significant release of ^{125}I (0.5% of the total, compared with 21.5% in the presence of chondrocytes). To demonstrate that a plasmin-like enzyme is responsible for the breakdown of fibrin, the concentration of acid-treated FCS was varied over 0–20% in the presence of a 1/25 dilution of SEM obtained after 1 day. Degradation of fibrin was shown to be dependent on the presence of serum, release of radioactivity was $0.8\% \pm 0.2\%$ for cultures without serum, $5.1 \pm 1.0\%$ for those containing 5% serum and $12.9 \pm 2.4\%$ at a serum concentration of 20%. This requirement for serum to 'unmask' the fibrinolytic activity indicates that the proteinase responsible for fibrin degradation is plasmin and that the chondrocytes are releasing a plasminogen activator rather than another proteinase which directly degrades the fibrin and which would therefore act independently of the presence of serum.

Human synovium releases a factor which stimulates chondrocyte production of PGE and plasminogen activator

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The mechanisms by which destruction of the tissues (cartilage, tendons, ligaments and bone) of joints affected by rheumatoid arthritis occurs are still poorly understood. Damage to the articular cartilage is a key event in the development of disability and deformity. Until recently it was thought that destruction of cartilage was primarily due to the release of a variety of proteolytic enzymes (collagenase and other neutral proteinases, and possibly lysosomal hydrolases) from the proliferating synovial pannus tissue¹. Little attention has been paid to the involvement of chondrocytes themselves in the destruction of cartilage. Recently Fell and Jubb² have shown that when porcine synovium and cartilage are maintained in the same organ co-culture system, destruction of the cartilage matrix occurs. They suggested that, in addition to inducing direct enzymatic breakdown of cartilage, synovial tissue might release a factor which activates chondrocytes to degrade their surrounding matrix. Steinberg *et al.*³ have confirmed that synovium and cartilage interact in this way in tissue culture, with breakdown and release of proteoglycans in cartilage. The purpose of our present study was to examine whether this interaction between synovium and cartilage occurs with human tissue and to examine the mechanisms in more detail. We have, therefore, investigated the effects of normal and rheumatoid synovial tissue on chondrocytes derived from human articular cartilage. We find that the culture medium from explants of normal and rheumatoid human synovium contains a factor which stimulates the production of prostaglandin E (PGE) and plasminogen activator by chondrocytes.

Normal synovium (~1.5 g) was dissected from human knee joints obtained after surgical amputation for trauma or lower limb ischaemia. The synovial tissue was dissected into small pieces and placed in explant culture in 20 ml of Eagle's modified essential medium (MEM) with 10% (v/v) fetal calf serum (FCS) and penicillin plus streptomycin. After 1 day the medium was changed and reduced to 15 ml, with subsequent changes and collections at days 1, 6, 9, 13 and 16. Medium was stored at -20°C . Chondrocytes were incubated with a 4% dilution of this conditioned synovial explant medium (SEM). PGE was assayed by radioimmunoassay in this medium and in the medium obtained after culture with chondrocytes. Since the antibody used detects PGB as well as PGE₁ and PGE₂, the media were subjected to silica gel microchromatography with the result that

the prostaglandin produced in chondrocyte cultures was shown to be predominantly of the E-type⁴.

To measure the release of plasminogen activator by chondrocytes, monolayer cultures were prepared using the method of Srivastava *et al.*⁵, with the following modifications: fragments of cartilage were incubated in hyaluronidase (1 mg ml⁻¹ in Dulbecco's Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline) for 15 min, then in 0.25% trypsin for 30 min, and then digested overnight with bacterial collagenase [*Clostridium* peptidase, (Sigma type I) 3 mg ml⁻¹ in MEM + 10% FCS]. Primary cultures were grown to confluence in MEM + 10% FCS, then subcultured after dislodging cells by exposure to trypsin. The cells were placed in multiwell dishes (16 mm diameter) coated with ^{125}I -fibrin⁶. Release of ^{125}I from the fibrin gel into the culture medium indicated the presence of plasminogen activator.

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Our results indicate that a factor is produced in the synovial explant cultures which, when added to chondrocytes, stimulates the production of PGE (Fig. 1a). The medium obtained after one day of culture with SEM increased the production of PGE by about 50-fold and the stimulatory effect diminished with continued culture of the synovium so that after 16 days of culture the SEM produced only a five-fold elevation of PGE levels when added to chondrocytes. Figure 2 shows that the response of the chondrocytes to produce PGE is dependent on the concentration of the SEM added. Addition of dexamethasone to chondrocytes, together with SEM, suppressed this activation of prostaglandin biosynthesis (Fig. 3). Medium from explants of rheumatoid rather than normal synovium was added to chondrocytes in other experiments and exhibited similar stimulatory effects.

Figure 1b shows that the same SEM also markedly stimulated the production of plasminogen activator by chondrocytes. The stimulatory effect on the production of plasminogen activator also seemed to diminish the longer the synovium was kept in culture. The similarities in the profiles of activation shown in Fig. 1a and b suggest that a single stimulatory factor might be involved and that there may be a close functional relationship between the production of prostaglandin and plasminogen activator.

In addition to showing that the human synovium produces a substance(s) which stimulates chondrocytes in culture, our present experiments demonstrate the hitherto unrecognized ability of chondrocytes to produce relatively large quantities of prostaglandin in culture. Our preliminary results indicate that protein synthesis is required for production of the factor since treatment of rheumatoid synovium with cycloheximide ($1 \mu\text{g ml}^{-1}$) resulted in a 75% reduction in production of stimulatory activity (21.75 ± 2.29 as compared with 80.25 ± 5.07 ng PGE per $100 \mu\text{g}$ cell protein per 24h in the dialysed medium control. The SEM was dialysed before addition to the chondrocyte cultures to eliminate the cycloheximide).

Further work is needed to characterize the source and nature of the stimulating activity, which may be similar to the factor, named catabolin, partially characterized by Dingle *et al.*⁷ This protein, as released from porcine synovium in culture, has a molecular weight of approximately 20,000 and stimulates the breakdown of cultured bovine nasal cartilage. There is also a similarity to the mononuclear cell factor described by Dayer *et al.*⁸ which stimulates production of PGE and collagenase by synovial cells and which we have found also to stimulate production of PGE by chondrocytes⁹. We have detected stimulatory activity from cultures of both normal and rheumatoid synovium which suggests that the factor is unlikely to be derived from inflammatory cells, which would only be present in appre-

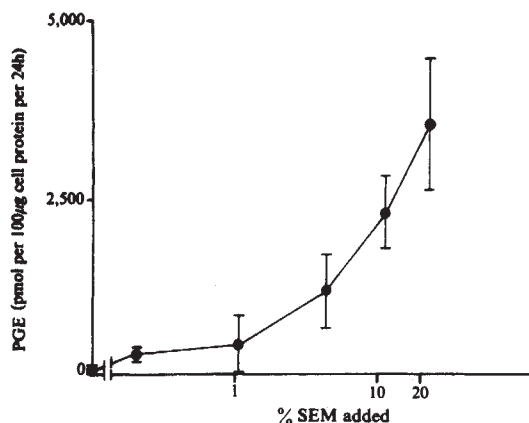


Fig. 2 The effect of SEM on PGE production by chondrocytes. The PGE originating from the SEM has been subtracted from each point. SEM collected at day 1 was incubated with chondrocytes for 24 h at dilutions between 1:250 and 1:5 (to give final concentrations of SEM in the culture fluid ranging from 0.4% and 20% as shown on the abscissa). Control cultures contained no SEM. The points shown are mean $\pm$ standard error of quadruplicate wells.

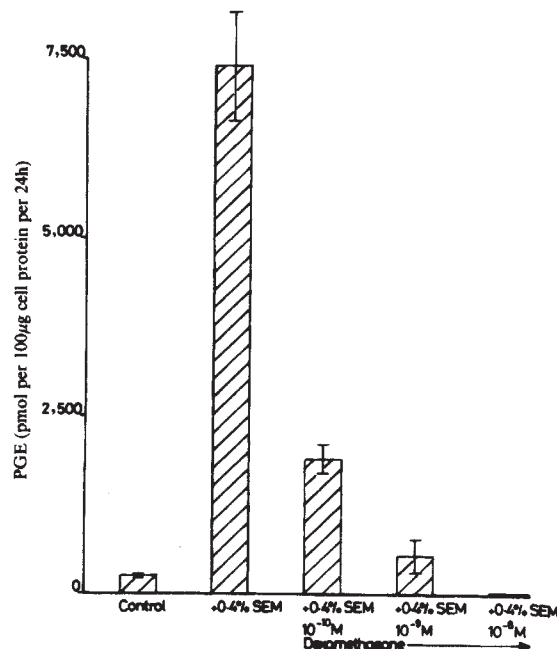


Fig. 3 Inhibition by dexamethasone of PGE production by human articular chondrocytes. Results are expressed as mean $\pm$ standard error of quadruplicate wells. Cultures did not contain FCS. SEM was collected on days 0–4 of culture and incubated with chondrocytes for 24 h. Controls did not contain SEM. PGE produced by the synovial explants has been subtracted from each point.

ciable numbers in pathological tissue. There are obvious similarities with a factor from rabbit macrophages which acts on rabbit chondrocytes to produce collagenase and other proteases^{11,12}.

The release of this factor from synovium could be of considerable significance in the pathogenesis of joint disease. Enhanced production of prostaglandins by chondrocytes could contribute to the increased PGE concentration found in synovial fluids in inflammatory arthritis¹⁰. PGE derived from chondrocytes could alter vascular permeability in adjacent tissues and could also stimulate resorption of the subchondral bone. These prostaglandins might also influence the rate of proliferation of the synovial pannus and be involved in controlling the mobility and activity of inflammatory cells. The plasminogen activator released by chondrocytes could itself degrade cartilage matrix by direct proteolytic attack or could take part in the conversion of collagenase from latent to active form. This factor from synovial tissue could therefore be responsible for some aspects of the tissue damage seen in joints in rheumatoid arthritis. The mechanisms that prevent such a factor from acting in normal conditions and which might allow its release in pathological circumstances require further investigation. The remarkable potency of added steroid in inhibiting the action of the factor on cartilage suggest that this may be one way in which steroids exert their therapeutic action in rheumatoid arthritis.

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Biosynthesis of hepatitis B virus surface antigen in *Escherichia coli*

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Hepatitis B is a widespread viral disease¹. In the absence of cell cultures capable of propagating the virus (HBV) an efficient vaccine has been prepared from viral envelopes isolated from the plasma of chronic carriers²⁻⁴. The major polypeptide of the envelope is one of molecular weight 25,000 which carries the surface antigen (HBsAg)⁵⁻⁸. Therefore, the biosynthesis of this polypeptide in *Escherichia coli* may offer an alternative procedure to produce HBsAg free from human proteins. Recently, the HBV genome has been cloned in *E. coli*⁹⁻¹¹. Determination of its primary structure allowed the localization of the gene (called gene S) coding for HBsAg¹²⁻¹⁵ and the synthesis of the core antigen in *E. coli* has been reported⁹. We have constructed a derivative of bacteriophage lambda carrying a fusion between the β -galactosidase gene (*lacZ*) and the HBsAg coding sequence (*l*acHBs-1). Infection of *E. coli* with *l*acHBs-1 leads to the biosynthesis of a polypeptide of molecular weight 138,000 carrying antigenic determinants of HBV surface antigen.

Expression of an eukaryotic gene in *E. coli* can be obtained by a fusion between the foreign gene and a bacterial gene carried by a plasmid or a phage. Thus the *lacZ* gene has already been used for hormone biosynthesis^{16,17}. Sequential steps in the construction of the gene fusion between gene S and gene *lacZ* are illustrated in Fig. 1. By *HhaI* treatment of the previously cloned HBV DNA¹⁰, we prepared a 1,084 base pair long DNA fragment which carried the entire gene S. Using *S*₁ endonuclease and *EcoRI* linkers 5'GGAATTCC, this fragment was cloned in *E. coli* using the plasmid pBR322 (ref. 20). The recombinant plasmid pBRHBs has only one *XbaI* restriction site located at the beginning of gene S. Digestion of this plasmid with *EcoRI* plus *XbaI* endonucleases, followed by a purification using agarose gel electrophoresis gave a DNA fragment of about 980 base pairs carrying a major part of gene S. After treatment with *S*₁ endonuclease, addition of *EcoRI* linkers, and cleavage with *EcoRI* endonuclease, this 980 base pair DNA fragment was cloned in *E. coli* using the plasmid pBR322. Several clones were obtained; the DNA of three clones (pXbaHBs-1, pXbaHBs-2 and pXbaHBs-3) was extracted and the 980-base pair *EcoRI* restriction fragment, called HBs-fragment, was purified in each case. Determination of the nucleotide sequence of the *EcoRI* extremity corresponding to gene S revealed that the nucleotide sequence was not identical in the three clones (Fig. 1A). The differences were possibly due to heterogeneity in the *S*₁ endonuclease digestion. The bacteriophage λ plac5-1UV5 genome¹⁹ has only one *EcoRI* site located at the end of the *lacZ* gene. The reading frame of this gene at the *EcoRI* site can be deduced from the amino acid sequence of the β -galactosidase²¹. Based on the nucleotide sequence, the insertion of the HBs fragment from pXbaHBs-1 into the *EcoRI* site of the *lacZ* gene of λ plac5-1UV5 genome should lead to conservation of the reading frame of gene S. The recombinant bacteriophage should

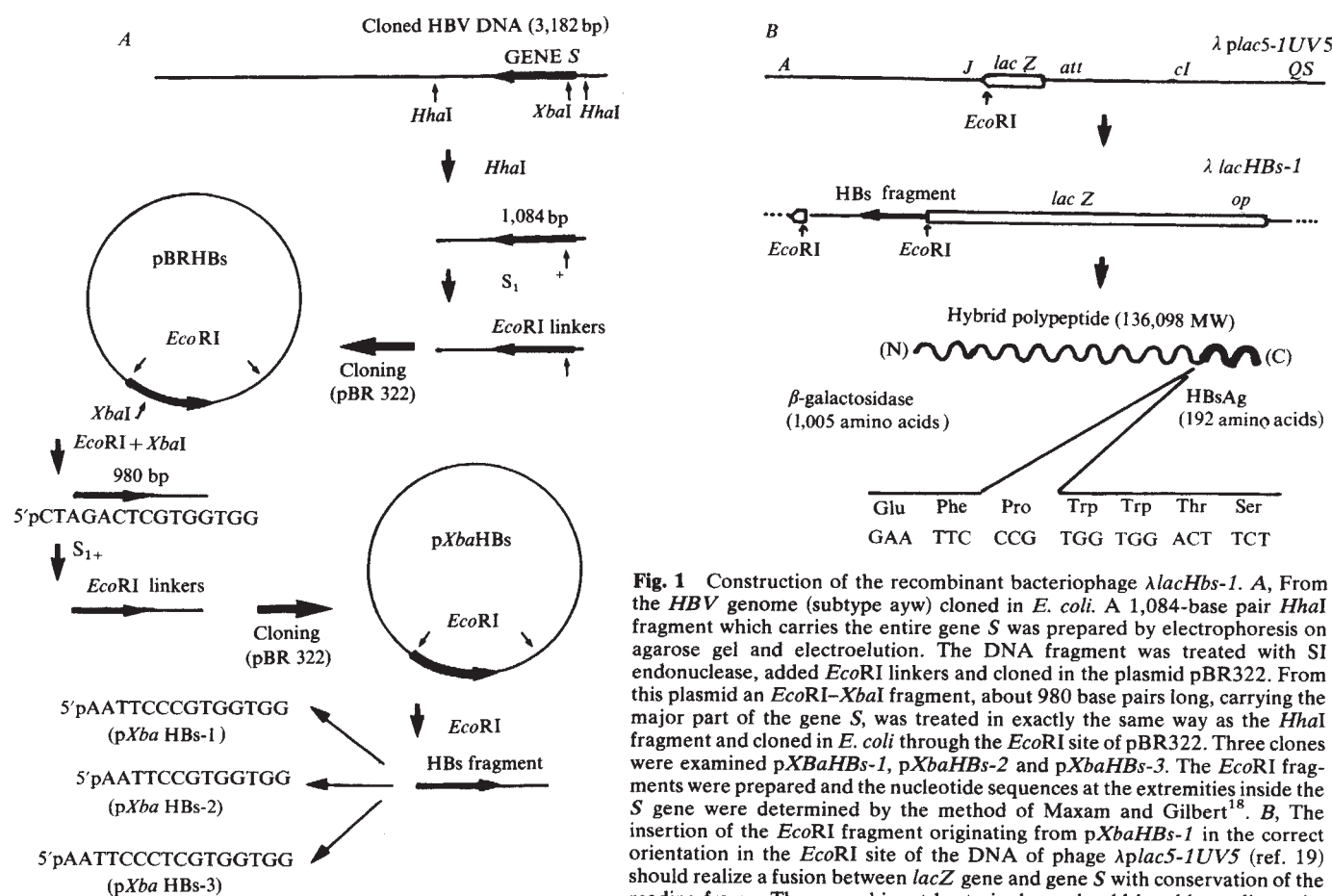


Fig. 1 Construction of the recombinant bacteriophage λ lacHBs-1. **A**, From the HBV genome (subtype ayw) cloned in *E. coli*. A 1,084-base pair *HhaI* fragment which carries the entire gene S was prepared by electrophoresis on agarose gel and electroelution. The DNA fragment was treated with *S*₁ endonuclease, added *EcoRI* linkers and cloned in the plasmid pBR322. From this plasmid an *EcoRI*-*XbaI* fragment, about 980 base pairs long, carrying the major part of the gene S, was treated in exactly the same way as the *HhaI* fragment and cloned in *E. coli* through the *EcoRI* site of pBR322. Three clones were examined pXbaHBs-1, pXbaHBs-2 and pXbaHBs-3. The *EcoRI* fragments were prepared and the nucleotide sequences at the extremities inside the S gene were determined by the method of Maxam and Gilbert¹⁸. **B**, The insertion of the *EcoRI* fragment originating from pXbaHBs-1 in the correct orientation in the *EcoRI* site of the DNA of phage λ plac5-1UV5 (ref. 19) should realize a fusion between *lacZ* gene and gene S with conservation of the reading frame. The recombinant bacteriophage should be able to direct the synthesis of a hybrid polypeptide of molecular weight 136,098 with a NH₂ terminal portion consisting of almost the entire β -galactosidase and a COOH terminal portion consisting of a great part of the HBsAg major protein.

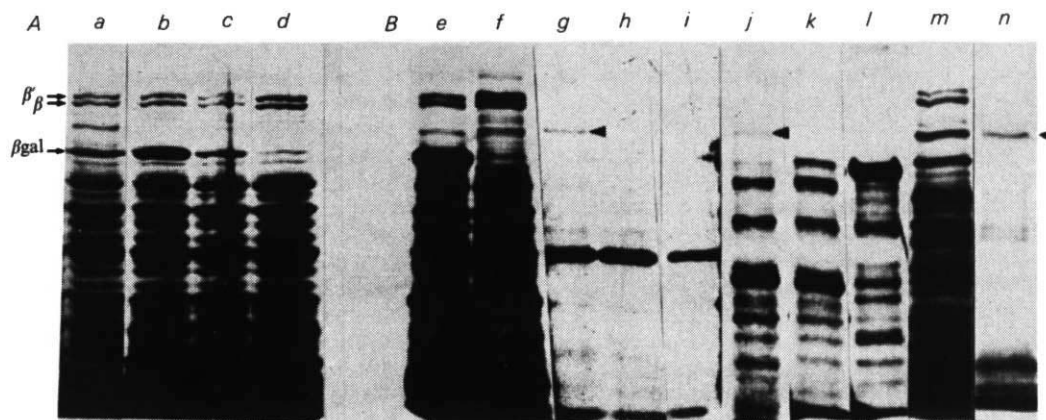


Fig. 2 Immunoprecipitation of the β -galactosidase-HBsAg hybrid protein. **A**, *E. coli* K12 3,000X74 was grown in Laemmli medium and concentrated to an absorbance of 10 units in MgSO_4 , 10 mM. Phages were absorbed for 15 min at a multiplicity of infection of 4. Cells were diluted 10 times in Laemmli medium and incubated 25 min at 37°C, centrifuged, resuspended in Laemmli final sample buffer²³ and boiled for 5 min. The proteins were electrophoresed on a 6% polyacrylamide-SDS gel²³ and stained with Coomassie blue. The proteins from cells infected with the following bacteriophages are shown: *a*, λ lacHBs-1; *b*, λ plac5-1UV5; *c*, λ lacHBs-2; *d* contains an extract of uninfected 3,000X74. The arrows show the position of size markers: β -galactosidase²¹ and β , β' subunits of RNA polymerase²⁴. **B**, Infected cells were diluted 10 times in Laemmli medium (Bacto-Tryptone: 10 g, Bacto yeast extract: 5 g, NaCl: 10 g in 1 L pH 7.3) containing [³⁵S] methionine (1050 Ci mmol⁻¹, 150 $\mu\text{Ci ml}^{-1}$) and incubated for 25 min at 37°C. Cells were centrifuged, resuspended in one-tenth volume (Tris pH 8: 10 mM, EDTA: 5 mM, lysozyme: 0.5 mg ml⁻¹, PTSF: 30 $\mu\text{g ml}^{-1}$) and lysed by three freeze-thaw cycles. The suspension was then made up to 10 mM MgCl_2 and 20 $\mu\text{g ml}^{-1}$ DNase was added; it was incubated for 15 min in ice and centrifuged. The supernatant was used for immunoprecipitation as follows: to 80 μl of this *E. coli* extract were added 120 μl TNT buffer (Tris pH 7.5: 50 mM, NaCl: 150 mM, Triton X100 1%) and 2 μl antiserum and the mixture was incubated for 60 min at 37°C. Then, 100 μl of a suspension of Protein A-Sepharose CL-4B (Pharmacia) in TNT buffer (100 mg ml⁻¹) was added and incubation was continued for 1 h at 0°C. The beads were pelleted by a 3 min centrifugation in an Eppendorf microfuge and then washed once with 500 μl TNT buffer, three times with 500 μl LiCl buffer (LiCl 500 mM, Triton X-100 1%, SDS 0.1%) and twice with 500 μl Tris pH 8: 10 mM. Finally, radioactive proteins were eluted from the beads by boiling for 5 min in 40 μl Laemmli final sample buffer²³. They were electrophoresed on a 6% polyacrylamide-SDS gel²³ which was then treated for fluorography²⁵, dried and subjected to autoradiography with flash-activated Kodak Royal X-Omat films at -70°C (ref. 26). The figure shows the proteins from cells infected with the following bacteriophages: *e*, λ plac5-1UV5; *f*, λ lacHBs-1; *g*, λ lacHBs-1, immunoprecipitation with human antiserum to HBsAg (subtype ayw) (generous gift from Dr J. Drouet); *h*, λ lacHBs-1, immunoprecipitation with the same antiserum to HBsAg in presence of HBsAg 100 $\mu\text{g ml}^{-1}$; *i*, λ plac5-1UV5, immunoprecipitation with the same antiserum to HBsAg; *j*, λ lacHBs-1, immunoprecipitation with rabbit antiserum to β -galactosidase (gift from Dr N. Guiso); *k*, λ lacHBs-2, immunoprecipitation with antiserum to β -galactosidase; *l*, λ plac5-1UV5 immunoprecipitation with antiserum to β -galactosidase; *m*, λ lacHBs-1QQS (cells are incubated 90 min at 37°C after infection); *n*, λ lacHBs-1QQS, immunoprecipitation with a sheep antiserum to purified HBsAg of ad and ay subtypes (gift from Dr J. Pillot).

be able to direct the synthesis of a hybrid polypeptide of molecular weight 136,098 (Fig. 1B). The HBs fragment from pXbaHBs-2 did not have a nucleotide sequence in the correct reading frame and it could be used as a control in further experiments. The HBs fragments from pXbaHBs-1 and pXbaHBs-2 were ligated with λ plac5-1UV5 DNA previously cleaved with *Eco*RI endonuclease. The mixture was used to transfect *E. coli* strain C600recBCrk⁻mk⁻. Since the insertion of a DNA fragment into the *Eco*RI site inactivates the *lacZ* gene²², the *lac*⁻ bacteriophage clones were purified on bacterial strain LA101²². The DNA of different bacteriophages was extracted and the orientation of the inserted DNA fragment was determined by electrophoretic analysis of the *Bam*HI restriction fragments. Two phage derivatives λ lacHBs-1 and λ lacHBs-2 corresponding to the plasmids pXbaHBs-1 and pXbaHBs-2 respectively had the HBs fragment in the correct orientation.

We infected strain 3,000X74 (Hfr Δ lacX74, Institut Pasteur Collection) with λ plac5-1UV5, λ lacHBs-1 and λ lacHBs-2. The bacterial protein lysates were analysed by polyacrylamide-SDS gel electrophoresis²³ and Coomassie blue coloration (Fig. 2A). The comparison of the three electrophoretic patterns revealed two major differences: in the case of λ plac5-1UV5, a much more intense band was observed at a position corresponding to β -galactosidase (molecular weight 116,248) mobility²¹; in the case of λ lacHBs-1 a new protein species (P138) of molecular weight 135,000–141,000 was detected. This protein was not present after infection with λ lacHBs-2.

Proteins synthesized after λ lacHBs-1 and λ plac5-1UV5 infection were labelled with [³⁵S] methionine. Precipitation with human antiserum to HBsAg followed by autoradiography of the polyacrylamide-SDS gel revealed a band present in the case of infection with λ lacHBs-1 and absent in the case of λ plac5-

1UV5 (Fig. 2B). This band disappeared specifically when the immunoprecipitation was carried out in presence of cold HBsAg. The precipitated protein co-migrated with the P138 protein. The same band was observed after immunoprecipitation with a sheep antiserum to purified HBsAg. A band was also present at the same position after immunoprecipitation with an antiserum to β -galactosidase (Fig. 2B).

These results demonstrate that *E. coli* infected with the bacteriophage λ lacHBs-1 synthesizes a protein of approximate molecular weight 138,000 with antigenic determinants of both HBsAg and β -galactosidase. These characteristics are exactly those predicted for the fusion polypeptide. The quantity of hybrid polypeptide was estimated by scanning the stained gels. It represents about 10% of the quantity of β subunit of the RNA polymerase, and about 1.5% of the quantity of β -galactosidase obtained after infection with λ plac5-1UV5. Assuming that the β subunit represents less than 0.7% of the total cell protein²⁷ the quantity of the hybrid protein should account for approximately 0.05% of total cell protein. An explanation for the low amount of hybrid polypeptide obtained could be proteolysis. Indeed, when the bacteria are lysed in the absence of protease inhibitor, the amount of P138 protein is still lower. Nevertheless, the introduction of amber mutations into genes *Q* and *S*^{22,28} of λ placHBs-1 genome, by genetic crossing with the bacteriophage λ plac5imm434Qam501Sam7 (ref. 22) enhances the P138 polypeptide production by a factor of approximately five (Fig. 2B). The β -galactosidase-HBsAg hybrid polypeptide provides a model for coupling HBsAg with a carrier protein in an immunoreactive form ultimately useful in newer vaccine against HBV infection²⁸. Moreover, the results presented reinforce the hypothesis that there is no intervening sequence in the HBsAg coding sequence^{12,13}.

Containment conditions for this study were recommended by the French National Control Committee. Growth of recombinant bacteriophages and plasmids was carried out in L3 conditions. We thank Drs P. A. Cazenave, D. Perrin, J. Thèze and G. N. Vyas for helpful discussions, J. Shapiro for reviewing the manuscript, and Drs A. Drouet and J. Drouet for critical suggestions and for providing antisera and HBsAg.

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Different target antigens for T-cell subsets acting synergistically *in vivo*

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After allogeneic transplantation of lymphoid cells into immunologically incompetent recipients, a graft-versus-host (GvH) reaction can occur. The original observation that the GvH reaction is mediated by two interacting types of T cell¹ has led to the proposal that T cells must be subdivided into two subpopulations according to life span and migratory properties. These consist of T₁ cells, which are short-lived, sessile cells sensitive to adult thymectomy (ATx), and T₂ cells, which are long-lived, recirculating cells sensitive to anti-thymocyte serum (ATS)². T₁–T₂ cooperation has also been demonstrated *in vitro* in murine³ and rat⁴ mixed lymphocyte culture. The question arises as to whether these T-cell subpopulations are activated by different or identical parts of the major histocompatibility complex (MHC). We report here that for optimal development of the anti-host immune response in a murine GvH reaction, T₂ cells have to be amplified by T₁ cells. The former cells are activated by a set of MHC gene products that are expressed mainly on immunological cells (H-2I-coded antigens), whereas the latter recognize a different set of MHC gene products that are expressed on almost all cells of the mouse (H-2K/D-coded antigens).

A delayed type hypersensitivity (DTH) assay was used to measure the development of specific anti-host directed T-effector cells⁵. The generation of these T-effector cells is induced by the host histocompatibility (H) antigens during a GvH reaction. Therefore, spleen and lymph node cells from mice which have been irradiated and reconstituted with allogeneic lymphoid cells are transferred intravenously (i.v.) into normal secondary recipients syngeneic to the original spleen cell donor. In these secondary recipients, the anti-host DTH reactivity is assayed by challenging the animals with the specific antigen and measuring the specific DTH response 24 h later.

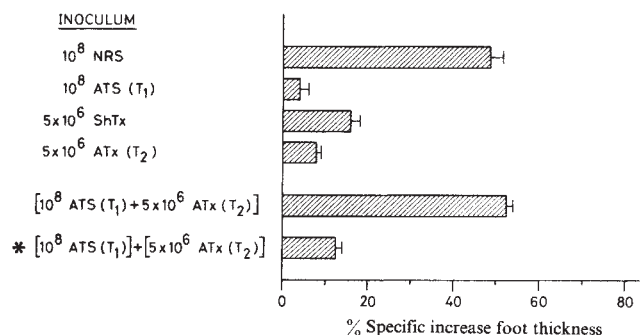


Fig. 1 GvH-related DTH responsiveness of spleen cells of lethally irradiated (C57BL/Rij × CBA/Rij)F₁ mice (H-2^{b/a}) reconstituted with T₁ and/or T₂ cells from DBA/2 mice (H-2^d). The DBA/2 donors were either treated *in vivo* with ATS (or NRS as a control) or were adult thymectomized (ATx) (or sham-thymectomized (ShTx) as a control) 6–8 weeks previously. ATS spleen cells were used as a source of T₁ cells, and ATx spleen cells as a source of T₂ cells. Five days after irradiation and reconstitution, the spleen cells of each recipient were i.v. transferred into a naive DBA/2 mouse. An asterisk means that T₁ and T₂ cells were separately injected into different lethally irradiated (C57BL × CBA)F₁ recipient mice. In that case, the spleen cells from both types of primary recipients were simultaneously transferred into the secondary DBA/2 recipients. All secondary recipients were challenged into the instep of the right hind foot with 2 × 10⁷ (C57BL × CBA)F₁ spleen cells. These spleen cells were treated with mitomycin C (Kyowa Hakko Kogyo), 100 μg ml⁻¹ for 30 min at 37 °C. At 24 h after challenge, the anti-host DTH reactivity was measured as the difference in thickness of the hind feet. The relative specific increase is calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of control mice, which had only received the challenge injection. The swelling of these control mice was 12–20%. DTH responses are expressed as the specific percentage increase in foot thickness. Horizontal bars represent 1 s.e.m. (n = 5).

For the experiments reported here, T₁ cells were obtained by pretreatment of spleen cell donor mice with ATS *in vivo*. This pretreatment gives an enrichment of T₁ cells in the spleen by selective depletion of the pool of recirculating T₂ cells⁶. Long-lived T₂ cells were obtained either from the spleen of donors 6–8 weeks after ATx, or by isolation *in vivo* of recirculating, lymph node-seeking T₂ cells according to the method of Tigelaar and Asofsky⁷.

We have previously shown that spleen cell transplantation across H-2 differences into lethally irradiated recipients did result in a transferrable anti-host DTH response^{5,8}. After transplantation of as much as 10⁸ ATS-treated DBA/2 spleen cells, as a source of T₁ cells, into lethally irradiated (C57BL × CBA)F₁ mice, no significant anti-host DTH-response developed (Fig. 1). In contrast, such a reactivity did appear after transplantation of 5 × 10⁶ spleen cells from ATx DBA/2 mice as a source of T₂ cells (Fig. 1). In this combination, simultaneous transplantation of T₁ cells and T₂ cells gave a much higher anti-host DTH response than the T₂ cells only. Anti-Thy-1.2 treatment of cell suspensions containing either T₁ or T₂ cells could completely prevent amplifier and effector cell activity,

Table 1 The target H-2-subregion coded antigens for T₁ and T₂ cells

Inoculum	Recipient	H-2 subregion coding for immunizing antigen*	DTH response
10 ⁸ C57BL NRS spleen	B10.G	K+I+D	25.9±1.4
10 ⁸ C57BL ATS spleen (T ₁)	B10.G	K+I+D	-1.8±3.2
1 LNS eq. C57BL (T ₂)	B10.G	K+I+D	8.1±1.4
10 ⁸ C57BL ATS spleen (T ₁)+1 LNS eq. C57BL (T ₂)	B10.G	K+I+D	25.2±2.3
10 ⁸ B10.AQR NRS spleen	B10.A×B10.T(6R)	K+I	33.4±2.6
10 ⁸ B10.AQR ATS spleen (T ₁)	B10.A×B10.T(6R)	K+I	3.5±2.5
1 LNS eq. B10.AQR (T ₂)	B10.A×B10.T(6R)	K+I	11.4±2.0
10 ⁸ B10.AQR ATS spleen (T ₁)+1 LNS eq. B10.AQR (T ₂)	B10.A×B10.T(6R)	K+I	26.8±1.2
10 ⁸ B10.AQR NRS spleen	B10.T(6R)	I	34.4±3.3
10 ⁸ B10.AQR ATS spleen (T ₁)	B10.T(6R)	I	-0.9±1.8
1 LNS eq. B10.AQR (T ₂)	B10.T(6R)	I	19.6±2.9
10 ⁸ B10.AQR ATS spleen (T ₁)+1 LNS eq. B10.AQR (T ₂)	B10.T(6R)	I	20.4±1.6
10 ⁸ A.TL NRS spleen	A.TH	I	31.2±2.1
10 ⁸ A.TL ATS spleen (T ₁)	A.TH	I	0.1±2.1
1 LNS eq. A.TL (T ₂)	A.TH	I	16.5±1.9
10 ⁸ A.TL ATS spleen (T ₁)+1 LNS eq. A.TL (T ₂)	A.TH	I	15.1±2.0

GvH reactions were elicited by i.v. injection of the inoculum into lethally irradiated recipient mice. Recipients were lethally irradiated (700 rad) within 4 h before reconstitution. At 5 days after reconstitution a number of cells equivalent to the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes of a recipient mouse were transferred i.v. to a secondary recipient syngeneic to the original spleen cell donor. Lymphoid cells from all primary recipients of a particular combination were pooled before secondary transfer. Immediately after transfer to the secondary recipients, these recipients were challenged in the right hind foot with 2×10^7 spleen cells syngeneic to the original irradiated recipients. At 24 h after challenge of the secondary recipients, DTH responses were measured. Numbers represent the arithmetic mean $\pm$ 1 s.e.m. of five mice. In view of the different donor-recipient combinations, the improvement of the T₂-cell-mediated DTH reactivity by admixing T₁ cells should be judged separately within each of the four different combinations. T₁ cells were obtained by pretreatment of donor mice subcutaneously with 0.2 ml ATS (or normal rabbit serum (NRS) as control). This amount was equally distributed over the inguinal and axillary areas. These injections were given 5 and 2 days before collecting the spleen cells. T₂ cells were isolated *in vivo*. 15×10^7 pooled spleen, peripheral and mesenteric lymph node cells of a particular donor strain were i.v. injected into syngeneic recipients which had been lethally irradiated 2 h previously. At 24 h after inoculation, only the peripheral lymph nodes were obtained from these recipients. Such peripheral lymph nodes contained about 4×10^6 more nucleated cells than lymph nodes from lethally irradiated, non-reconstituted mice. The cell yield from all peripheral lymph nodes of a single irradiated recipient is called one lymph node seeking equivalent (1 LNS eq.).

* H-2 subregion difference between inoculum donors and irradiated recipient mice. The origin of H-2 subregions (K, I-A, I-B, I-C, D) for the strains are as follows: A.TH, ssssd; A.TL, skkkd; B10.A, kkkdd; B10.AQR, qkkdd; B10.T(6R), qqqqd; C57BL, bbbbbb; B10.G, qqqqq.

respectively⁹. When T₁ and T₂ cells were separately activated by H antigens and thereafter combined and simultaneously transferred into secondary recipients, no synergistic reactivity was observed (Fig. 1). This indicates that cooperation of T₁ and T₂ cells only occurs during the induction phase of the anti-host immune response and not during the inflammatory reaction in the challenged foot of the secondary recipients.

In Table 1, upper part, T₁-T₂ synergism is shown for the anti-host DTH response of C57BL lymphoid cells against B10.G mice (which differ at the K, I and D subregions of the H-2 complex), and after transplantation of B10.AQR lymphoid cells into (B10.A×B10.T(6R))F₁ mice (which differ at the K and I subregions). Also in these combinations, simultaneous transplantation of T₁ and T₂ cells gave a much higher anti-host DTH response than the T₂ cells alone. Because the anti-host DTH response after transplantation across a whole H-2 difference is directed exclusively to H-2I-coded antigens⁸, and H-2K/D antigens by themselves are unable to evoke an anti-host DTH response¹⁰, these results might suggest that T₁ cells are activated by H-2K and/or H-2D region-coded antigens, and amplify the anti-H-2I response by T₂ cells. This hypothesis was evaluated by testing the occurrence of T₁-T₂ synergism during the development of an anti-host DTH response in the case of transplantation across a H-2I difference only. This was done by transplanting T₁ and T₂ cells from B10.AQR mice into lethally irradiated B10.T(6R) mice and from A.TL mice into irradiated A.TH recipients. In both situations, no evidence for T₁-T₂ synergism could be found (Table 1, lower part). Thus, T₁ cells must be activated by H-2K/D antigens to display amplifier activity. The formal proof of this conclusion would be a positive response after transfer of mixtures of the two T-cell subpopulations after separate activation of the T₂ cells by I-coded antigens and of the T₁ cells by K/D-region coded antigens. However, such experiments are impossible because simultaneous transfer of separately activated T₁ and T₂ cells does not result in any detectable synergistic activity (Fig. 1).

The consequence of the differential specificity of receptors of T₁ (anti H-2K/D) and T₂ (anti-H-2I) cells is that both subpopulations must belong to different, parallel lines of T-cell

differentiation. Recent observations^{11,12} suggest that there are two separate lines of intra-thymic T-cell differentiation with regard to Lyt phenotype, one line leading to Lyt 1⁺ T cells and the other to Lyt 123⁺ T cells. The latter cells might further differentiate into Lyt 23⁺ cells after their migration as Lyt 123⁺ cells to the periphery^{12,13}. A part of this post-thymic Lyt 123⁺ population is ATx sensitive and may be identical to the T₁ population¹⁴. The data presented here indicate that post-thymic T₁ cells (probably Lyt 123⁺ cells), on stimulation by K- and/or D-region coded antigens, can amplify the generation of anti-host DTH effector cells from T₂ cells (probably Lyt 1⁺ cells), activated by I-region coded antigens. Such T₂-derived effector cells can release factors which lead to the expression of GvH-related DTH on transfer (in our system) or to help in humoral responses. The present results lead us to speculate that in the case of enhancement of T₂-helper cell activity by T₁-amplifier cells^{15,16}, both subpopulations are restricted by different H-2 subregions—T-helper cells by H-2I and T-amplifier cells by H-2K/D.

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An immunoglobulin heavy-chain gene is altered in two T-cell clones

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Thymus-derived lymphocytes (T cells) show a high degree of discrimination in their responses to various antigens, very similar to the specificity repertoire of antibody-producing B cells. The nature of the T-cell receptor which mediates antigen recognition is obscure, but the ability to discriminate between antigenic specificities implies a range of receptor specificities. Many serological and genetic data suggest that T-cell receptors use the immunoglobulin heavy (H)-chain variable (V) region genes but do not carry the antigenic determinants of the immunoglobulin H-chain constant (C) regions; they also do not seem to carry conventional light (L)-chain V- or C-region determinants¹⁻³. In B cells and derivatives the expression of immunoglobulin genes is manifested, at the DNA level, by an alteration of the restriction enzyme patterns of both the H and L immunoglobulin genes⁴⁻⁹. Specifically, V-gene integration involves joining of a V gene with a J segment (in the case of the H chain probably through an intermediate D segment)^{4,6,7} so that sites for restriction enzymes will undergo changes in cells in which V-J joining has occurred. Here, we describe Southern filter hybridization experiments¹⁰ using $C\mu$ and $C\kappa$ probes on the DNA of individual T-cell clones from mice, and present evidence for alteration of sequences adjacent to the $C\mu$ gene in the cells.

The T-cell clones used in this study were two killer cell lines (AD9 and HY72)¹¹. Both the cell lines were derived from stimulated splenic T cells and display an H-2-restricted cytotoxic activity against cells bearing H-Y antigen (an activity which has remained unchanged over several months of culture). The cells also express the Thy-1 surface antigen. The T cells were derived from different strains of mice and we have therefore compared the size of the restriction fragment containing the $C\mu$ gene in liver DNA from various mouse strains using a cloned $C\mu$ cDNA probe ($p\mu/118$)¹². Figure 1 shows the result of a Southern filter hybridization experiment between $KpnI$ digests of liver DNA and nick-translated $p\mu/118$. Considerable polymorphism can be seen between the different strains with respect to the $KpnI$ restriction fragment containing the $C\mu$ gene. We have also found polymorphism between γH -chain genes of different mouse strains but no differences in the $C\kappa$ gene of any of the strains studied (unpublished). A separate study has shown that the intervening sequence between V and $C\gamma 1$ regions of an IgG1-producing myeloma cell contains short tandemly repeated sequences which may also occur in regions adjacent to $C\mu$, to the other $C\gamma$ and to the $C\alpha$ genes¹³. It is possible, therefore, that the polymorphism in the size of restriction fragment containing the CH genes is a result of genetic instability within these tandemly repeats similar to, for example, the instability associated with the *Xenopus* rDNA tandemly repeated sequences¹⁴.

To detect rearrangement events around a J segment it is necessary to cleave the DNA to the 5' side of the J segments. $KpnI$ cleaves about 1.5 kilobases to the 3' side of the $C\mu$ gene¹⁵; therefore, the sizes of the $KpnI$ fragments (see Fig. 1 legend) of the different strains are all consistent with the possibility of a second cleavage site to the 5' side of the JH region, given that JH and $C\mu$ are separated by about 8 kilobases¹⁵. Figure 2 compares the hybridization of $p\mu/118$ ($C\mu$ probe) with $KpnI$ -digested T-cell DNA or liver DNA from the respective strain with a

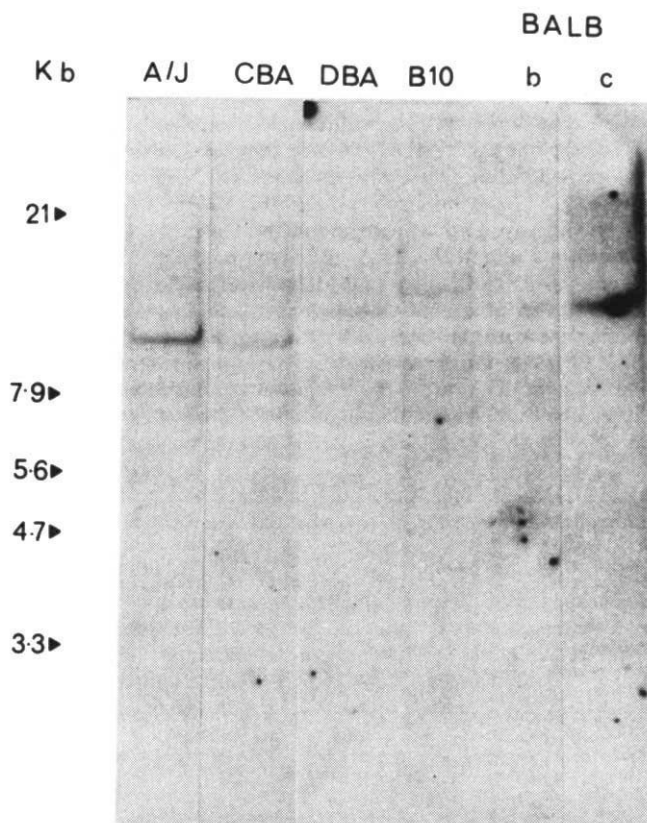


Fig. 1 Hybridization of $C\mu$ probe with liver DNA from various strains of mice. High molecular weight DNA was prepared from livers of A/J, CBA, DBA/2, B10A, BALB/c and BALB/b mice, digested to completion with $KpnI$ and 15- μ g aliquots fractionated on 0.8% agarose (alongside λ phage DNA digested with $EcoRI$ as size markers). The DNA was denatured and transferred to Schleicher and Schüll nitrate filters¹⁰. The $C\mu$ probe $p\mu/118$ is a cDNA clone containing all of the $C\mu$ coding region plus a substantial amount of 3' noncoding sequence¹², and was radioactively labelled by nick-translation (specific activity 4×10^7 c.p.m. per μ g). The filters were hybridized as previously described^{28,29} using $6 \times$ SSC salt conditions and 10^6 c.p.m. ml^{-1} of probe for 2 days at $65^\circ C$. The filters were washed after hybridization in $1 \times$ SSC salt conditions and autoradiography was carried out for 2 weeks at $-70^\circ C$ using prefogged film and an intensifying screen³⁰. Fragments found in BALB/c, BALB/b and DBA are around 14 kilobases (kb), about 15 kilobases in B10A DNA, 12 kilobases in CBA and 12.5 kilobases in A/J DNA.

myeloma cell line (McPc 1748) which produces IgM. In each case, the liver DNAs display only a single hybridizing band which presumably reflects the presence of a single $C\mu$ gene in these mice. Compared with the liver DNA, DNA from the myeloma line (Fig. 2a) and the killer T-cell clones (Fig. 2b, c) display new restriction fragments hybridizing to the $C\mu$ probe. The IgM-producing myeloma shows two new hybridization bands (about 20 and 18.5 kilobases), indicating that rearrangement events have occurred on both immunoglobulin-bearing chromosomes in this cell line. Unlike the myeloma DNA, T-cell DNAs possess only single new rearranged fragments containing a $C\mu$ gene as well as a fragment approximately coincident with that found in the liver DNA. Thus, it seems likely that these cells have undergone DNA alteration at the JH cluster but that this event has occurred on only one chromosome, in contrast to the IgM-producing cell line. We have also found evidence of similar rearrangements in a Moloney virus-transformed cell line (Thy-1 positive, A. D. Lowe, personal communication), but we could find no rearrangement in a cell line such as P815 (a mouse mastocytoma).

Furthermore, cleavage of the DNA with *EcoRI*, which cleaves between $C\mu$ and $JH^{9,15}$, obliterates the evidence of rearrangement (unpublished data), lending support to the hypothesis that the events involve the JH region.

We have examined the possibility of κ -chain rearrangement in the T-cell clones and in the IgM-producing clone using a similar strategy—digesting DNA with enzymes which cleave 3' of $C\kappa$ and 5' of $J\kappa$ (*BstI* and *EcoRI*). McPc 1748 (Fig. 3a) possesses two *EcoRI* fragments hybridizing to the $C\kappa$ probe (23 and 15 kilobases) of which the latter is common to the unique $C\kappa$ -containing *EcoRI* fragment of BALB/c liver, indicating that this cell line has an integrated $V\kappa$ - $C\kappa$ gene in one chromosome. The comparative hybridization of the $C\kappa$ probe to AD9 DNA and CBA liver DNA digested with *BstI* (Fig. 3b) shows only a common hybridization component (about 12.5 kilobases) which may be attributed to the unintegrated $C\kappa$ gene. Similar results were obtained with both HY72 DNA and the mastocytoma cell (P815) (unpublished). The absence of $C\kappa$ or $C\mu$ rearrangement in P815 DNA is consistent with results recently published by Kemp *et al.*¹⁶, who found no evidence of $C\kappa$ or $C\mu$ transcripts in P815 RNA.

The data presented are, therefore, consistent with the conclusion that DNA changes have occurred which are associated with sequences to the 5' end of the $C\mu$ gene but that κ -gene rearrangements have not occurred in the T-cell clones studied. These changes around the $C\mu$ gene, by analogy to the results in B cells, could be due to VH - JH joining or may be due

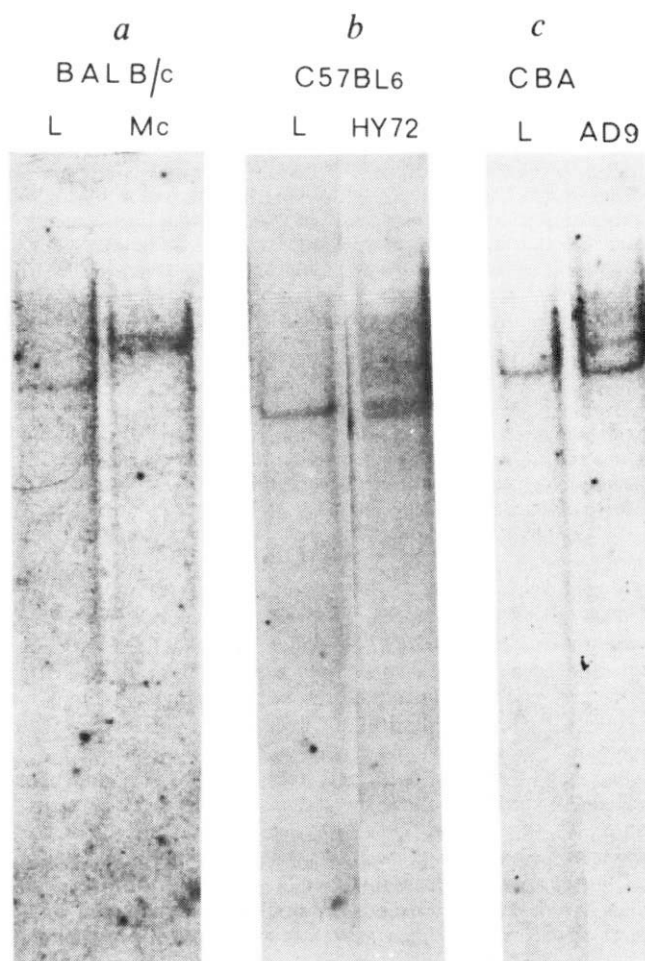


Fig. 2 Profiles of T-cell and B-cell DNA hybridization with $C\mu$ probe. High molecular weight DNA of BALB/c liver (L) and McPc 1748 cells, C57BL/6 liver and HY72 cells, and CBA liver and AD9 cells were digested to completion with *KpnI*, fractionated and hybridized as in Fig. 1 with $\mu/118$. a-c Represent separate fractionations of DNA.

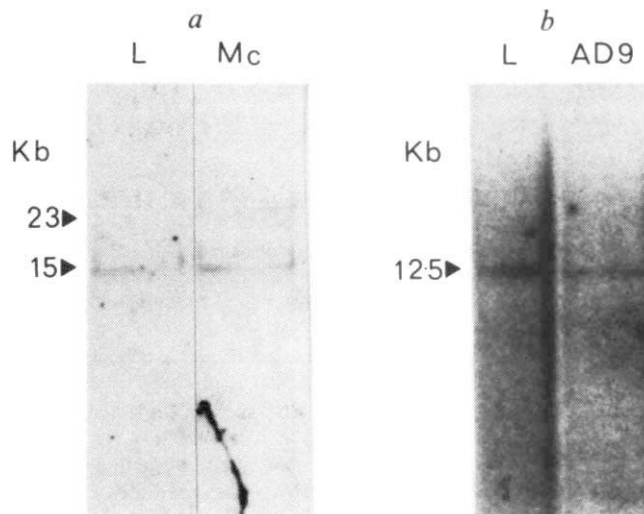


Fig. 3 Hybridization of mouse DNA to $C\kappa$ probe. The various high molecular weight DNAs were completely digested with *EcoRI* (panel a) and *BstI* (panel b), fractionated and transferred to cellulose nitrate filters as in Fig. 1. The $C\kappa$ probe was isolated from the cloned cDNA $\rho\kappa(11)^{24}$ (ref. 31) by digestion with *HpaI*: this enzyme cuts once in the pMB9 vector and once within the $C\kappa$ region of the insert near the V/C junction³² to yield two fragments of which the 3.7-kilobase fragment contains the $C\kappa$ region. The fragment was isolated from a preparative agarose gel and labelled by nick-translation. The filters were hybridized and washed as in Fig. 1. a, L = BALB/c liver DNA; Mc = McPc 1748 DNA. b, L = CBA liver DNA; AD9 = AD9 killer T-cell DNA.

to an as yet unknown DNA activity in this area. Although the T-cell and B-cell DNA may share these rearrangement features around the $C\mu$ gene, they differ in that the T-cell DNAs show no evidence of $C\kappa$ -gene rearrangement. Although the results presented here do not necessarily imply T-cell expression of this region of the immunoglobulin locus, there is evidence that $C\mu$ genes are expressed in thymocyte population or T-cell lymphomas^{16,17}. The present study utilizes cloned T-cell lines as a source of DNA rather than tumour lines or thymus cells.

One can speculate on possibilities which could reconcile these results with what is known of T-cell receptors at the cellular level and H-chain genes at the molecular level. It seems that deletion of DNA between VH and CH genes occurs during the H-chain class switch^{8,18-20}. Thus, as the T cells possess $C\mu$ genes, it can be inferred that a putative constant region sequence of the T-cell receptor (C_T) would need to be located between the VH cluster and the $C\mu$ gene or within the $C\mu$ gene itself. In the mouse, JH segments and the $C\mu$ genes are separated by about 8 kilobases of intervening sequence¹⁵ which could easily accommodate a constant-region gene. A second possibility is that C_T is generated from the $C\mu$ gene, partly from the $C\mu$ coding reading frame sequence and partly from new reading frames, or new combinations of coding sequences created by differential splicing, such as occurs in the formation of the SV40 and polyoma virus T antigens²¹⁻²³ or as is now known for the formation of the membrane form of μ -heavy chains^{24,25}. A third possibility is that C_T is located on a separate chromosome which can recombine with the immunoglobulin locus to translocate VH to C_T or vice versa.

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Projection patterns of single physiologically characterized optic tract fibres in cat

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Because the axons of retinal ganglion cells are the sole channels carrying information from the eye, the organization of their central projections is important in visual processing. However, their detailed destinations and patterns of synaptic distribution at the level of single, functionally identified cells are not known. Most anatomical studies involve populations of cells or fibres and do not examine their physiological properties; physiological studies involving intracellular recording and injection of marker substances into cell bodies^{1–3} of single cells do not reveal distant axon terminals because the markers stain the fibres for only a few millimetres from the perikarya. To examine the central projections of retinal ganglion cells we have impaled single optic tract fibres near their sites of termination^{4,5} and injected them iontophoretically with the marker enzyme horseradish peroxidase (HRP). We now report that this method has revealed the thalamic and midbrain ramifications of single physiologically characterized axons. The individual optic-tract fibres branch repeatedly, sending collaterals to the superior colliculus (SC), the medial interlaminar nucleus (MIN), and to one or more laminae within the dorsal lateral geniculate nucleus (LGNd). In different nuclei the single axons form arborizations of characteristically different shapes and distribute their synaptic terminals in columns (LGNd), sheets (MIN) or widely spread patches (SC).

We used standard procedures for studying visual physiology in mammals⁶, for HRP histochemistry^{7,8} and for morphological reconstruction. To maximize transport of the HRP out of the electrode tips we used high enzyme concentrations and large d.c. currents. The electrodes were filled by centrifugation with a pre-centrifuged solution of 200 mM potassium acetate (pH 6.8) and 10% HRP. The tips were broken to diameters of about 0.5 μ m, giving d.c. resistances of 200–500 M Ω . To protect their tips and guide their descent to the optic tract, the electrodes were lowered from the surface of the cortex inside a stereotactically positioned glass micropipette (Horsley–Clarke ant. 6,

lat. 9; see Fig. 1a). The membrane potentials recorded from the axons were 10–40 mV negative with positive-going action potentials of 2–10 mV amplitude. On completing physiological testing of an impaled axon, we injected it with HRP using a current of 20–50 nA (electrode-positive) for 30–200 s. During the injection the current was periodically turned off and action potentials from the axon were observed to confirm that it remained impaled. The length of stained axon varied with the total injected charge and appeared complete in the geniculate and colliculus with 5–10 μ C. At their injection sites the axons appeared swollen and were surrounded by extracellular debris (see Fig. 1b). Several hundred micrometres away the axons appeared normal and had diameters, including myelin, of 2–5 μ m. Results reported here are based on 9 of 14 axons we recorded and injected in the optic tracts of four adult cats. All nine axons (three from ipsilateral and six from contralateral eyes) were functionally classified as Y-type based on standard criteria including high conduction velocities^{9,10} (30–40 m s⁻¹) and nonlinear spatial summation in their receptive fields^{11,12}.

Figure 2 represents a frontal view of the left thalamus and midbrain showing the reconstruction of an on-centre optic tract axon from the contralateral eye. The axon was impaled (arrow) in the optic tract just ventral to the LGNd. The centre of the axon's receptive field was 1° in diameter and was located on the vertical midline 13° below the projection of the area centralis. The axon was spontaneously active, gave transient responses to steps of light, and responded well to rapidly moving stimuli. Based on a contrast reversal test¹³, the effects of light were nonlinearly summed in its receptive field. The axon's retinal origin and its high conduction velocity (latency 0.36 ms) were demonstrated by electrical stimulation with a hook electrode placed near the head of the optic nerve.

Each of the nine axons sent collaterals to three nuclei: the LGNd, the MIN and the SC. In each nucleus the collaterals formed arborizations with characteristic patterns (see Fig. 2): converging to single loci in the LGNd, diffuse and radiating in the SC, and spread out in thin sheets in the MIN. Thus, individual ganglion cells have structurally different patterns of arborization at each of their nuclear destinations. Each axon gave rise to a total of 1,000–2,000 synaptic-like boutons which appeared in the sections as spherical or irregular swellings 0.5–3 μ m in diameter. Such swellings have been identified in the LGNd as synaptic terminals¹⁴. The axon shown in Fig. 2 had about 1,400 of these terminals: 225 in the SC, 1,134 in the LGNd and 34 in the MIN.

In the LGNd the single axons from the contralateral eye always terminated in both laminae A and C. This is illustrated by

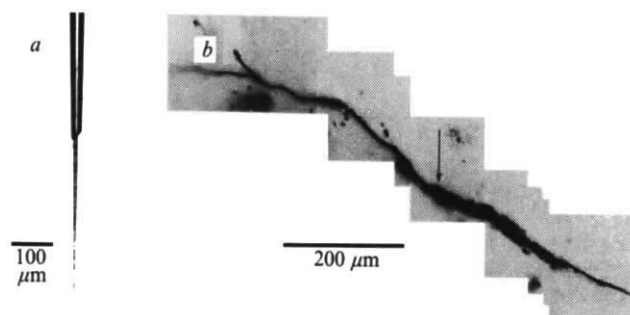


Fig. 1 a, Photograph of the glass micropipette used to protect and guide the tip of the electrode into the optic tract. The recording microelectrode extends out of the pipette. The micropipette was made from 1.6 mm o.d. glass pulled to a taper of 25 mm and broken to a tip diameter of about 30 μ m. The electrodes were made with 30-mm tapers so that they could extend several millimetres out of the micropipette. b, Photomicrograph montage showing detail of the axon of Fig. 2 at its injection site in the optic tract. The axon passes from the upper surface of the 100- μ m section (lower right) to the lower surface (out of the picture at far left). It branches and sends a collateral back to the upper surface of the section (upper left). The arrow indicates the site of injection based on a line projected from the electrode track.

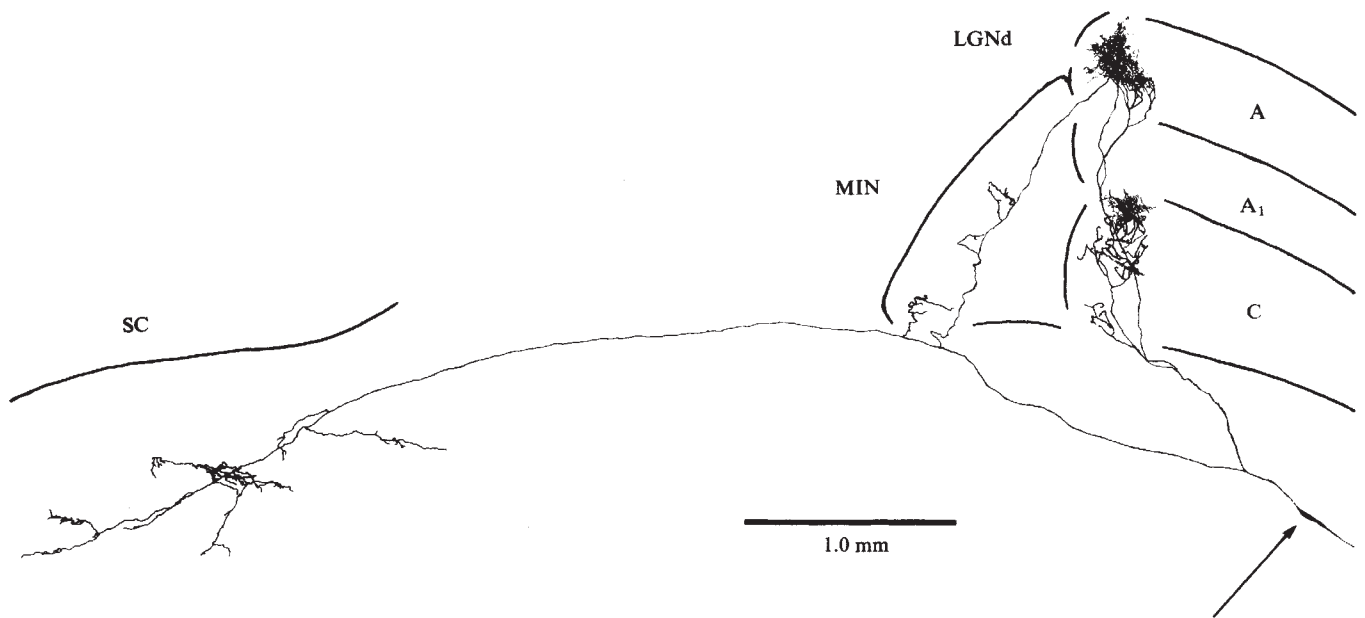


Fig. 2 Reconstruction from coronal sections of a single on-centre Y-type axon showing its terminations in the LGNd, MIN and SC. The arrow indicates the site of injection into the axon (see Fig. 1*b*). The figure represents over 3 mm of depth in the anterior-posterior axis (perpendicular to page) so that the perspective is flattened and the shapes of the nuclei and laminae are not representative of a single coronal plane. The arborization in the SC is in the anterolateral part of the nucleus.

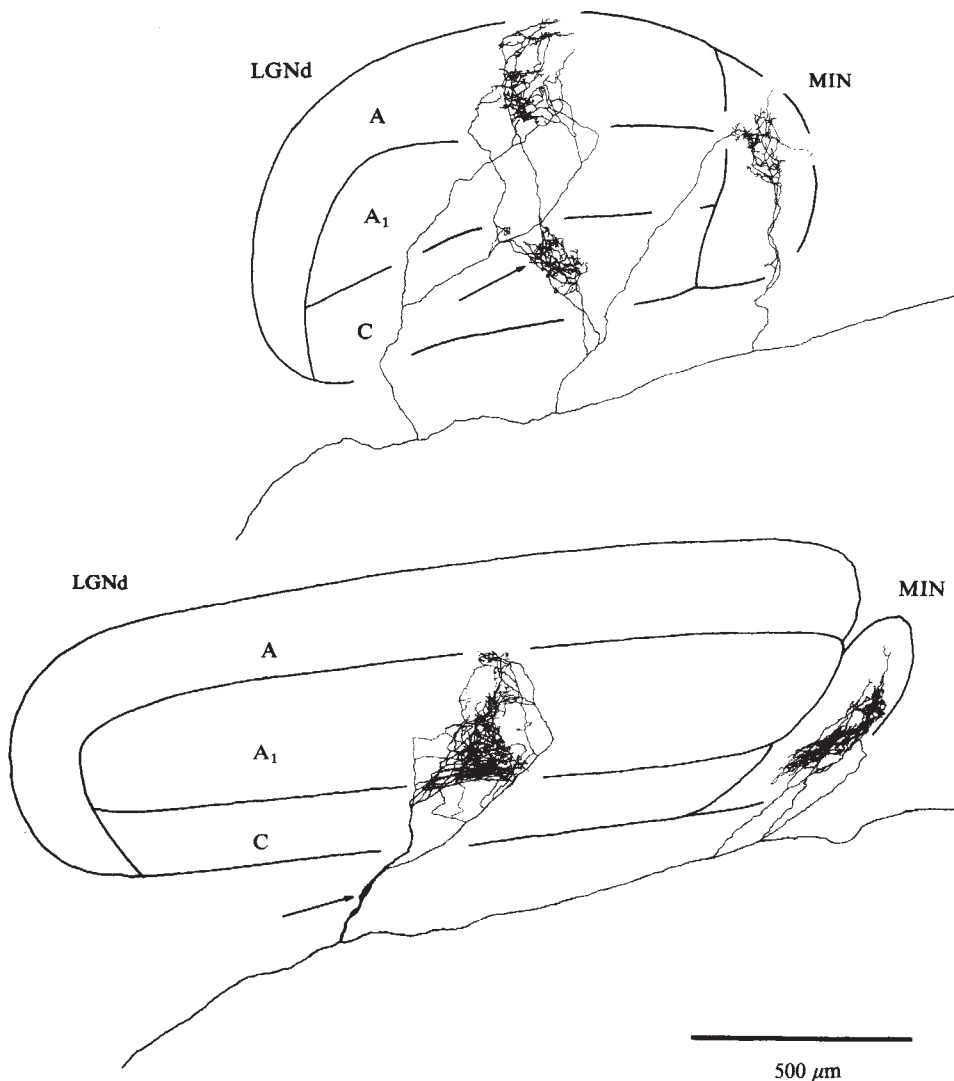


Fig. 3 Reconstructions of single axons showing their terminations in the right LGNd and MIN. The shapes of the terminal distributions in the MIN are bowl-like (concave toward the LGNd) and extend in the anterior-posterior axis about as far as they do in the plane of the figure. Arrows indicate injection sites. Upper: on-centre Y axon from the contralateral eye with a 4.5° receptive field centre located 28° below the horizontal meridian and 26° from the vertical midline. Lower: off-centre Y axon from the ipsilateral eye with a 4.5° receptive field centre located 1° above the horizontal meridian and 8.5° from the vertical midline.

the axons of Figs 2 and 3 (top). In at least two cases (for example see Fig. 2), these axons terminated in all three of the contralateral laminae (A, C, C₂). Whether this was always the case is not clear because the borders of C₂ were not always evident in our sections¹⁵. Axons from the ipsilateral eye terminated in only a single lamina. They arborized in A₁ but not in the other lamina (C₁) that receives ipsilateral input (see Fig. 3, bottom).

In three instances in which the sections were cut at right angles to the planes of the laminae, the synaptic terminals were seen to be distributed in broad-based columns that spanned the thickness of lamina A or A₁. Figure 4 shows one of these columns reconstructed from the axon of Fig. 3 (bottom). Each dot represents the position of a single terminal in A₁. The columns provide an anatomical basis for the physiologically observed 'projection columns'¹⁶ which run through the nucleus. As with the projection columns at the same locations, the columns of terminals were tilted forward with respect to vertical¹⁷. Their broad bases, resulting in a greater density of Y terminals in the ventral portions of the laminae, may account for the apparent segregation of Y afferents that has been seen in field potential recordings¹⁸. The shape of the columns shows that there is an intra-laminar gradient in the spatial distribution of synaptic connections across the laminae. The functional significance of this gradient is unknown.

In the planes of the geniculate laminae, which correspond to the plane of the retinotopic map, the distributions of the terminals were basically circular. The maximum lateral spread was constant (300–400 μ m) in spite of the fact that the receptive fields ranged from 1° to 4.5° in diameter and were located between 8° and 38° from the area centralis. This constancy of spread is consistent with the idea¹⁷ that variations in geniculate receptive field sizes result from response properties of the input fibres rather than from anatomical differences within the nucleus. It also suggests uniformity and repetition in the geniculate, a plan of organization similar to that in the striate cortex, and one that may have developmental advantages¹⁹.

In the MIN the retinotopic map is a mirror image of that in the LGNd²⁰. Each axon distributed its terminals in the MIN as a sheet 300–400 μ m across and about 50 μ m thick. The sheets were slightly concave toward the LGNd, conforming to the general curvature of the MIN. They were orientated approximately perpendicular to the geniculate laminae. The form of the sheets can be appreciated from Fig. 3, taking into account that the perspective is distorted by projection onto a flat surface. The spread of the terminals in the plane of the MIN map (about 50 μ m) was a factor of six to eight times less than the spread of the terminals in the plane of the geniculate map, a factor which is of the same order as the overall size difference between the MIN and LGNd. Thus, on the basis of the lateral spread of afferent terminals, the nuclei seem to be scaled similarly.

The synaptic terminals in the SC were in the superficial layers of the nucleus. A dorsal view of the SC arborization from Fig. 2, obtained by rotating the reconstruction graphically, revealed that the terminals were grouped into irregular patches which were distributed as an ellipse (1 × 2 mm) with its major axis orientated approximately medio-laterally. It would be interesting to know exactly how these patches are positioned relative to the columnar organization described in the SC for ipsilateral eye afferents and acetylcholinesterase activity²¹. It is clear that the major axis of distribution was approximately orthogonal to the columns and must have spanned several of them.

In the geniculate complex single axons often had two or more long branches which travelled along different paths to converge on the same retinotopic location (see especially Figs 2 and 3, top). It is not clear what functional significance, metabolic constraint, or mode of development these multiple and somewhat indirect pathways may reflect. What they do show is that in the adult there is considerable freedom in the paths that an axon may take to its target.

A second and perhaps related feature was that the forms of the arborizations in the LGNd and MIN were often only a crude guide to the spatial distribution of the axons' terminals. Some



Fig. 4 Columnar distribution in lamina A₁ of terminals from the axon of Fig. 3, bottom. Reconstructed from four 100- μ m serial sections. Number of terminals is about 1,200.

arborizations were diffuse and composed of processes that arose from branch points well outside the area of their actual terminations (compare Fig. 3, bottom, with the terminal distribution in Fig. 4). Other arborizations, such as the one in lamina A of Fig. 2, were dense and nearly congruent with the terminal distributions. The lack of close correspondence between the forms of the arborizations and the spatial distributions of the terminals was especially marked in the anterior–posterior axis of the LGNd. Here, sagittal sections showed that the arborizations were stretched out toward the posterior of the nucleus in 'cypress-like'²² fashion. The form of the arborizations and the multiple pathways of the axons' long branches may arise during development as a result of morphological changes following synaptogenesis in the target nuclei. In the developing cat geniculate, the formation of the laminae, as well as growth and rotation of the nucleus, occurs after the arrival of the retinal afferents²³. In the monkey geniculate similar events have been specifically shown to occur after synaptogenesis has begun²⁴. If the migrating cells drag their presynaptic axons with them, then the terminals could be carried far away from the axons' branch points. In contrast to their geniculate patterns, axons in the colliculus go directly to their sites of termination by single, short paths (see Fig. 2). In this regard it would be interesting to know how the sequences of developmental events and patterns of morphogenesis differ between the cat colliculus and geniculate.

The results reported here are based on a sample of one physiological class of retinal axons. It will be of interest to examine single axons of other functional types, such as those from X cells¹² which have linear spatial summation and from colour cells which have opponent spectral properties. The

experimental approach of staining single axons may also be useful in answering questions about axonal growth in developing preparations and in adult animals whose development has been altered by early experience, lesions or genetic factors.

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***In vivo* electrochemical detection of catechols in the neostriatum of anaesthetized rats: dopamine or DOPAC?**

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Electroanalytical techniques for the *in vivo* measurement of neurotransmitters in brain tissue have been applied especially to the catecholamines, which are easily oxidizable¹⁻⁶. Measurements are, however, complicated by the presence of ascorbic acid (AA) in brain tissue^{1,3-6}. Lane *et al.*⁷ have been able to circumvent this problem, at least in part, by the application of differential pulse voltammetry (DPV) to a surface-modified platinum electrode, obtaining distinct oxidation current peaks in recordings from the rat neostriatum which are attributed to AA and to dopamine (DA), respectively, but which are also unstable. We have recently described a new type of electrode^{2,8}, consisting of a pyrolytic carbon fibre 8 µm thick and 0.5 mm long. We now report that the DPV method used in conjunction with an electrochemical treatment of this electrode yields stable and reproducible peaks in which catecholamines and AA are resolved from each other. Moreover, pharmacological investigations suggest that the catecholamine peak measured *in vivo* in the rat neostriatum should be attributed to 3, 4-dihydroxyphenylacetic acid (DOPAC), suggesting that our technique may be a useful means of following dopaminergic activity *in vivo*.

A classical three-electrode system was used with a conventional Ag/AgCl reference electrode. The carbon fibre electrodes were prepared as described elsewhere⁸, and electrochemically treated by applying a triangular wave potential

(0-3 V, frequency 70 Hz) for 20 s, with the electrode tips immersed in phosphate-buffered saline (PBS). Differential pulse (DP) voltammograms (Polarograph Tacussel PRG5; scan rate 20 mV s⁻¹, pulse modulation of 20 mV per 0.2 s) were recorded *in vitro* in PBS containing 20 µM DOPAC and 200 µM AA until the signal remained stable (~15 min). Before and after implantation each electrode was calibrated in standard solutions of DA, DOPAC, AA and dihydroxyphenylalanine (dopa). AA peaked at -50 mV and catechols, such as DOPAC, DA or dopa, peaked at +100 mV (Fig. 1). They yielded linear current responses with increasing concentrations of AA (0-1 mM), DOPAC (0-60 µM), DA or dopa (0-2 µM). These linear responses were not affected by the *in vivo* utilization of the electrode. The minimum measurable signal corresponded to about 2 µM for DOPAC and 0.2 µM for DA or dopa.

Carbon fibre electrodes were implanted in the neostriatum of anaesthetized rats (chloral hydrate 400 mg kg⁻¹). DP voltammograms recorded each 2.5 min showed two peaks: 'peak 1' appeared at the same potential as AA *in vitro* (-50 mV); and 'peak 2' appeared at the same potential as the catechols (+100 mV) (Fig. 1). We have shown that peak 1 disappeared in the striatum of guinea pigs that had been fed on a vitamin C-deficient diet and have suggested that peak 1, obtained from the striatum, is therefore due to AA⁹. Unfortunately, peak 1 was often found to be distorted, showed considerable variance from one animal to another, and appeared to be a function of the depth of anaesthesia. Thus, further work is needed to find better conditions (different parameters for the treatment of the microelectrode, use of chronic implanted rats) that will allow a more precise determination of AA *in vivo*.

In contrast, peak 2 appeared very stable and reproducible (Fig. 2). After an unilateral and selective destruction of the nigrostriatal dopaminergic pathway (performed and controlled as previously described²), peak 2 was suppressed in the striatum where the dopaminergic terminals degenerated, but was still present on the intact side (Fig. 1). Moreover, administration of α -methyl-*p*-tyrosine, a specific inhibitor of catecholamine synthesis¹⁰, decreased the amplitude of peak 2 (Fig. 2). These results indicate that peak 2 represents the DP oxidation current of one or several catechol compounds, probably related to catecholaminergic nerve terminals in the striatum.

Several catechol compounds present in the striatum can be ruled out as being significant contributors to peak 2. Noradrenaline content in the striatum is about 1% that of DA¹¹

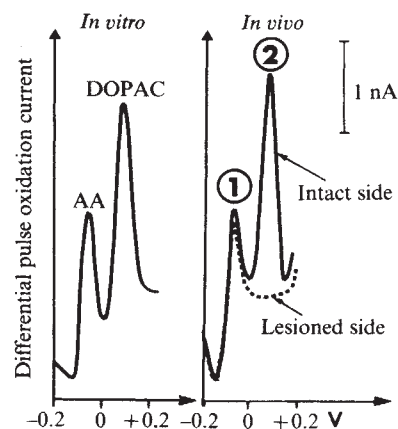


Fig. 1 Differential pulse voltammograms from *in vitro* experiments and from the neostriatum of anaesthetized rats (*in vivo*). Electrochemically treated carbon fibre electrodes were tested in PBS containing AA (200 µM) and DOPAC (20 µM). *a* Shows typical DP voltammogram. *b* Represents one typical example of DP voltammograms recorded in one rat with the same electrode implanted alternately in both striata. The lesioned side corresponds to a striatum in which the dopaminergic terminals were selectively destroyed following 6-hydroxydopamine injection in the substantia nigra². Peak 2 suppression was observed with all the lesioned rats (*n* = 6).

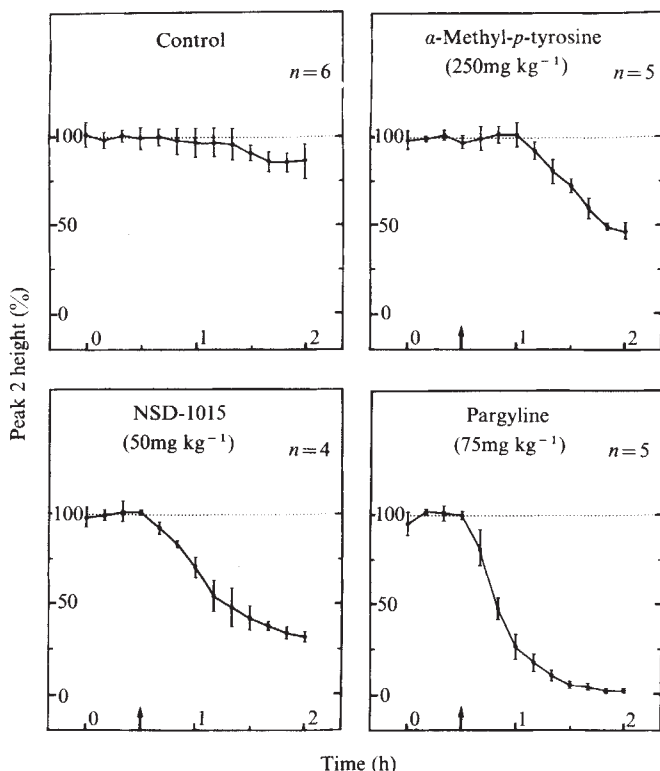


Fig. 2 Evolution of peak 2 height measured from DP voltammograms obtained from the neostriatum in control rats and rats treated with α -methyl-*p*-tyrosine (Sigma), NSD-1015 (Sandoz) and pargyline (Sigma). All animals were anaesthetized with chloral hydrate (400 mg kg^{-1} , i.p.) and kept anaesthetized with additional injections as needed. Arrows indicate that i.p. drug injections were performed 30 min after the beginning of the recordings. For each animal the results were expressed as the percentage of the mean control value calculated by averaging the four initial absolute values of peak 2 height (100%). These four initial baseline responses were estimated by comparison with DOPAC calibration curves obtained after the *in vivo* experiment: the mean average value for all experiments was $22 \pm 4 \mu\text{M}$ (mean $\pm$ s.d., $n = 33$). Results represent the mean $\pm$ s.d. of n rats.

and the contribution of noradrenaline and its metabolites to peak 2 is thus negligible compared to that of DA and its metabolites. O-methylated metabolites of DA, such as homovanillic acid and 3-O-methyldopamine, are oxidized at more positive potentials^{3,12}. dopa, DA and DOPAC are oxidized at +100 mV and consequently could contribute to peak 2 *in vivo*. They are present in the dopaminergic nerve terminals: DA is the neurotransmitter released from the nerve terminals, synthesized from dopa through dopa decarboxylase, and its major breakdown product due to monoamine oxidase activity is DOPAC. To elucidate which product was the major contributor of peak 2

the DA system was manipulated with drugs known to act at different levels of the dopaminergic metabolic pathway. Inhibition of dopa decarboxylase by NSD-1015 rapidly increases the striatal dopa concentration¹³. In our study, NSD-1015 induced a marked decrease of the peak 2 height (Fig. 2). Consequently, it is unlikely that dopa is responsible for the peak 2 recorded *in vivo*. Pargyline, which inhibits monoamine oxidase, is known to cause a rapid decrease in DOPAC^{14,15} and an increase in DA concentration¹⁶ in the striatum. Pargyline suppressed peak 2 with a half life of ~ 15 min, in agreement with previous observations^{15,17} and this supports the notion that DOPAC is the major contributor to peak 2.

This suggestion was confirmed by the results obtained with other drugs. In fact, reserpine and haloperidol, which are known to raise striatal DOPAC levels^{10,14,16,18}, induced a pronounced increase in the peak 2 amplitude (Fig. 3). Amphetamine, which markedly increases DA release¹⁰ and decreases the striatal DOPAC concentration^{14,16}, caused a diminution in peak 2 height. The effects of these three drugs were tested again in pargyline (75 mg kg^{-1} , intraperitoneally (i.p.)) pretreated rats (1.5 h before drugs). In these conditions, peak 2 disappeared completely and did not reappear after reserpine. After haloperidol and amphetamine (five animals each) a very small peak 2 reappeared which reached a maximum after about 40 min. Its amplitude was in all cases found to correspond to an estimated concentration $< 0.2 \mu\text{M}$ DA.

These results show that peak 2 is mainly due to DOPAC oxidation. For all drugs tested alone, the variations in amplitude and time course of peak 2 were consistent with previous studies of DOPAC concentrations measured using fluorimetric or radioenzymatic methods¹⁴⁻¹⁸. In our study the concentration of DOPAC in the striatum was calculated by comparing peak 2 heights *in vivo* and *in vitro*, and was estimated to be $22 \pm 4 \mu\text{M}$ (mean $\pm$ s.d., $n = 33$). This value is compatible with a global striatal tissue concentration of DOPAC under chloral hydrate anaesthesia of $7-15 \mu\text{M}$ refs 10, 14, 18).

A very slight contribution of DA to peak 2 was observed only after amphetamine or haloperidol. The very low DA levels thus measured are consistent with values found using the push-pull technique¹⁹. In the light of these observations and considering the size of the active part of the electrode, it can be assumed that events are recorded in the extracellular space occupying about 20% of the total tissue²⁰. The presence in the striatum of a potent DA inactivating process acting through high affinity uptake by nerve terminals²¹ prevents DA diffusing from the synaptic cleft to the extracellular space, thus contributing to maintaining in this space very low DA concentrations. This questions the interpretation of our previous results, suggesting that DA was responsible for the increase in the oxidation current measured in the striatum after amphetamine². However, these experiments were performed using a non-selective technique (pulse amperometry and untreated carbon fibre electrodes). In the present study the decrease in peak 2 amplitude observed after amphetamine treatment was always associated with a significant rise in peak 1 height, although important differences in the amplitude of this peak were observed.

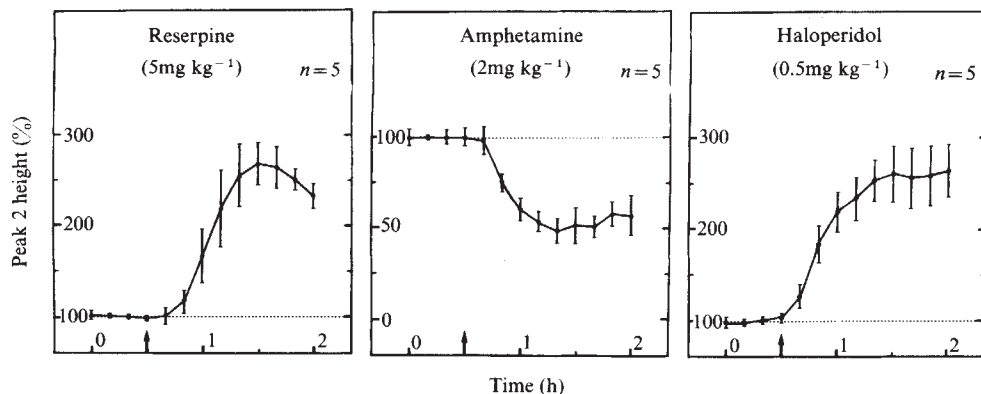


Fig. 3 Effect of reserpine (Serpasil, Ciba-Geigy), amphetamine (La Coopération Pharmaceutique Française) and haloperidol (Haldol, Le Brun) on peak 2 height measured on DP voltammograms recorded from the neostriatum of anaesthetized rats.

The electrochemical treatment used in this study improves the selectivity, sensitivity and reproducibility of the carbon fibre electrode. In conclusion, the present investigation clearly demonstrates that the catechol oxidation current peak obtained *in vivo* from the striatum of anaesthetized rats is DOPAC and not DA.

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Insulin triggers cyclic AMP-dependent activation and phosphorylation of a plasma membrane cyclic AMP phosphodiesterase

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Regulation of blood glucose levels by the liver is primarily achieved by the action of two peptide hormones, insulin and glucagon, which bind to specific receptors associated with the hepatocyte plasma membrane. Whilst the molecular action of glucagon at the level of the cell plasma membrane in activating adenylate cyclase is relatively well understood, we know little, if anything, of the molecular consequences of insulin occupying its receptor. We demonstrate here that insulin, at physiologically relevant concentrations, can trigger the cyclic AMP-dependent activation and phosphorylation of a low K_m cyclic AMP phosphodiesterase attached to the liver plasma membrane. Such an effect may in part explain the ability of insulin to inhibit the increase in cellular cyclic AMP content that glucagon alone produces by activation of adenylate cyclase. Our observation that basal, intracellular cyclic AMP levels are insufficient to allow insulin to activate the cyclic AMP phosphodiesterase, yet those cyclic AMP levels achieved after exposure of the cells to glucagon are sufficient, gives a molecular rationale to Butcher and Sutherland's proposal¹ that it is necessary to first elevate cellular cyclic AMP levels before they can be depressed by insulin.

The cyclic AMP phosphodiesterase activity of rat liver plasma membranes can be divided into a high K_m intrinsic enzyme exhibiting normal Michaelis kinetics and a low K_m peripheral

enzyme exhibiting apparent negative cooperativity². The low K_m enzyme is distinct from cytosol forms² and binds with high affinity to a protein site on the plasma membrane (R.J.M. and M.D.H., unpublished observations).

Treatment of purified rat liver plasma membranes with combinations of insulin, cyclic AMP and Mg^{2+} -ATP, in the conditions described in Fig. 1, resulted in a doubling of the cyclic AMP phosphodiesterase activity only when all three components were added and when native insulin was used. The activation attained maximal levels within 3 min (Fig. 1A) and was not reversed by washing the membranes with 1 mM $KHCO_3$, pH 7.4. The activation was entirely due to the peripheral, low K_m enzyme which could be released by high ionic strength treatment² in a 2.5-fold activated state when assayed at 4×10^{-7} M cyclic AMP (Fig. 1B). Relative to the native enzyme², the released, activated protein exhibited a slightly lower K_m , 0.59 μ M, and similar V_{max} , 1,140 pmol min⁻¹ mg⁻¹, but there was a significant reduction ($P < 0.01$) in the Hill coefficient from 0.59 ± 0.05 (s.d., $n = 4$) for the native enzyme² to 0.45 ± 0.03 (s.d., $n = 4$) after activation.

Dose-response curves for the insulin-mediated activation of the cyclic AMP phosphodiesterase yielded a K_a of 1.4×10^{-10} M (Fig. 2), demonstrating the effectiveness of physiological concentrations. Interestingly, the cyclic AMP dependence of this phenomenon, exhibiting a K_a of 1.6×10^{-6} M, indicates that, at basal intracellular cyclic AMP levels of 0.3–0.5 μ M (refs 3, 4), little if any activation would be triggered by insulin (Fig. 2). However, after exposure to glucagon, cyclic AMP levels rise to 2.0–4 μ M (refs 1, 5) which would allow activation by insulin (Fig. 2).

The dependence of insulin's activation of the cyclic AMP phosphodiesterase on both Mg^{2+} -ATP and cyclic AMP indicated that a cyclic AMP-dependent phosphorylation might be involved. Because cyclic AMP-dependent protein kinases may be blocked by a specific inhibitor protein acting at the cyclic AMP regulatory subunit⁶, we added such an inhibitor protein to the system and found that it prevented the activation of the cyclic AMP phosphodiesterase in a dose-dependent fashion (Fig. 1). Furthermore, polyacrylamide gel electrophoresis (PAGE) of the peripheral proteins of the liver plasma membrane after treatment with insulin, cyclic AMP and ^{32}P -ATP yielded three distinct peaks of radioactivity (Fig. 3). The major peak clearly migrated with the peripheral cyclic AMP phosphodiesterase activity. No ^{32}P from ^{32}P -ATP was incorporated into these peaks when (1) either or both cyclic AMP and insulin were absent, or (2) when a concentration of cyclic AMP protein kinase inhibitor protein just sufficient to prevent activation of the cyclic AMP phosphodiesterase was also present (Fig. 3). SDS-PAGE of the peripheral proteins obtained from liver plasma membranes pretreated with ^{32}P -ATP, insulin (10^{-9} M) and cyclic AMP (10^{-5} M) resolved three peaks of radioactivity. The major one, which is presumably the cyclic AMP phosphodiesterase, migrated with an apparent molecular weight of 52,000. From the estimates of molecular size of the enzyme in non-denaturing conditions², this would imply that it is a monomeric species. The two minor bands migrated with apparent molecular weights of 28,000 and 14,000. The dose-response curves for incorporation of ^{32}P into this major peak are given in Fig. 2a, b, where they demonstrate K_a 's for insulin and cyclic AMP which are identical to those exhibited for the activation of the phosphodiesterase by these ligands. Indeed, a K_a of $\sim 10^{-10}$ M for insulin's action is reflected in a similar affinity of insulin for specific binding to its receptor^{7,21}.

It is generally accepted and confirmed here (Fig. 1) that insulin alone does not activate the liver plasma membrane cyclic AMP phosphodiesterase of membrane fragments^{8–11} although there is consistent evidence that such an activation can occur in intact adipocytes⁸ and, in some instances, in intact hepatocytes^{8,9}. Our novel observation is that for insulin to achieve such an activation then cyclic AMP and Mg^{2+} -ATP must both be present at appropriate concentrations. This may explain insulin's

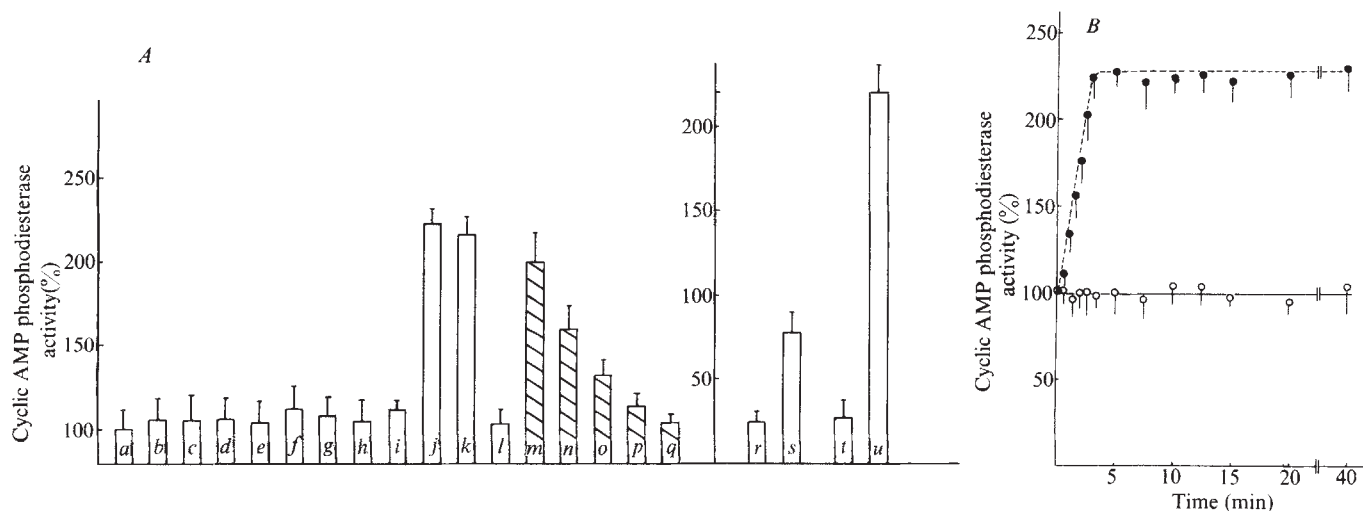


Fig. 1 *A*, Insulin requires ATP and cyclic AMP to stimulate the peripheral plasma membrane cyclic AMP phosphodiesterase activity. Purified rat liver plasma membranes^{2,11} (500 $\mu\text{g protein ml}^{-1}$) were incubated for 5 min at 30 °C in a cocktail with final concentrations of 5 mM MgCl_2 , 100 $\mu\text{M CaCl}_2$, 20 mM Tris HCl, pH 7.4, at a final volume of 250 μl . Various additions to the cocktail were made at the final concentrations stated and at a final pH 7.4. *a*, No additions; *b*, 3×10^{-3} M ATP; *c*, 10^{-9} M insulin; *d*, 10^{-6} M insulin; *e*, 10^{-6} M cyclic AMP; *f*, 10^{-4} M cyclic AMP; *g*, 3×10^{-3} M ATP + 10^{-6} M insulin; *h*, 3×10^{-3} M ATP + 10^{-4} M cyclic AMP; *i*, 10^{-6} M insulin + 10^{-4} M cyclic AMP; *j*, 10^{-6} M insulin + 10^{-4} M cyclic AMP + 3×10^{-3} M ATP; *k*, 10^{-9} M insulin + 4×10^{-6} M cyclic AMP + 3×10^{-3} M ATP; *l*, as in *j*, but using insulin denatured as described in ref. 13; *m-q*, as *j*, but a cyclic AMP-dependent protein kinase inhibitor, prepared as described in ref. 22, was added at final concentrations of *m*, 0.2 $\mu\text{g ml}^{-1}$; *n*, 0.4 $\mu\text{g ml}^{-1}$; *o*, 1.0 $\mu\text{g ml}^{-1}$; *p*, 1.0 $\mu\text{g ml}^{-1}$; *q*, 4.0 $\mu\text{g ml}^{-1}$. After this incubation the mixture was rapidly cooled on ice and then washed three times with 1 ml ice-cold 1 mM KHCO_3 , pH 7.4, by repeated centrifugation at 14,000g for 10 min at 4 °C with subsequent resuspension. Aliquots (40 $\mu\text{g protein}$) of intact plasma membranes were then assayed for cyclic AMP phosphodiesterase activity as described previously² using 4×10^{-7} M cyclic AMP as substrate. Routinely, a modified two-step radioassay based on that described by Thompson and Appleman²³ was used as we could demonstrate negligible adenosine deaminase activity contaminating our preparations², although similar results were obtained using the method of Rutten *et al.*²⁴. Corrections were made for adenosine bound to the resin². The activity of the intrinsic enzyme was identical for both *r* (native) and *t* (treated), whereas that of the native peripheral enzyme (*s*) was increased 2.5-fold in the treated case (*u*). Separation of the peripheral and integral enzymes was carried out as in ref. 2. Similar results were obtained using plasma membrane derived from hepatocytes as isolated in ref. 2. Errors are $\pm$ s.d., $n=6$ using six different plasma membrane preparations. *B*, Time course of the insulin-triggered, cyclic AMP-dependent activation of liver plasma membrane cyclic AMP phosphodiesterase. Liver plasma membranes were incubated in the cocktail described in legend to *A* either $\bullet$, with 10^{-9} M insulin + 4×10^{-6} M cyclic AMP + 3×10^{-3} M ATP, or $\circ$, without such additions. Reactions were terminated as described in *A* and samples taken for assay of cyclic AMP phosphodiesterase at 4×10^{-7} M cyclic AMP. Errors are $\pm$ s.d. for three membrane preparations ($n=3$). The initial activity of the cyclic AMP phosphodiesterase was 45 $\text{pmol min}^{-1} \text{mg}^{-1}$ in the assay conditions used. The control enzyme was unaffected by incubation in this cocktail over a period of 40 min.

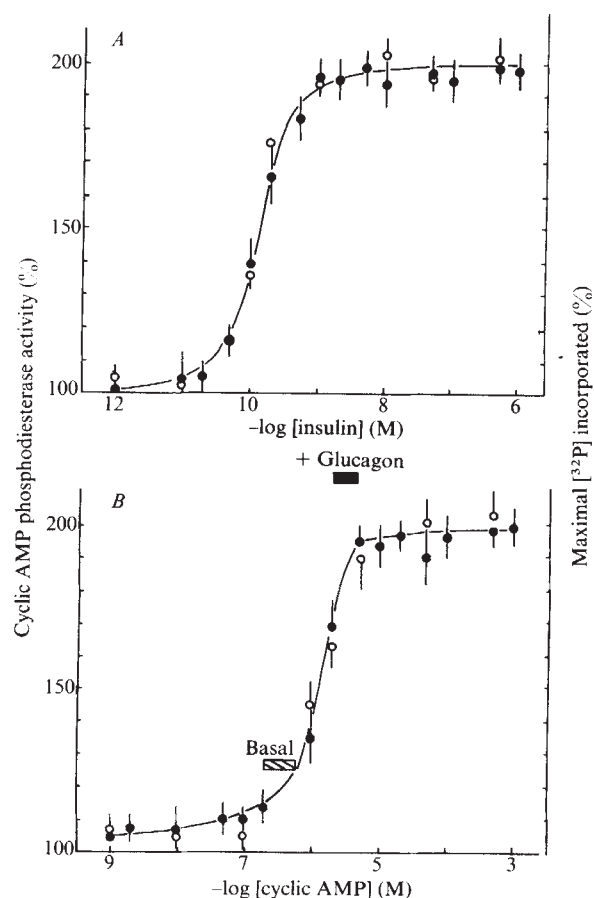


Fig. 2 Dose-response curves for the activation of the peripheral plasma membrane cyclic AMP phosphodiesterase. Incubations, assay of cyclic AMP phosphodiesterase and the release and separation of the peripheral enzyme after exposure to the stimulating ligand were carried out as described in Fig. 1A legend. For the assay of ^{32}P incorporation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the incubation mixture contained 1 mM ATP at a specific radioactivity of 200 mCi mMol^{-1} . The incorporation of radioactivity into the major peak found by SDS-PAGE of the peripheral proteins (see Fig. 3 legend) is plotted as a function of either insulin or cyclic AMP concentration. *A*, Dose-response curve for the activation of the peripheral cyclic AMP phosphodiesterase ($\bullet$) in the presence of saturating, 10^{-6} M, bovine insulin. Similar results were obtained using 10^{-9} M insulin. The ATP concentration was 3×10^{-3} M. The dose-response curve for the incorporation of ^{32}P into the major peak obtained upon SDS-PAGE of the peripheral proteins ($\circ$) was carried out at an insulin concentration of 10^{-9} M. *B*, Dose-response curve for the activation of the peripheral cyclic AMP phosphodiesterase ($\bullet$) in the presence of saturating, 10^{-4} M, cyclic AMP. Similar results were obtained using 4×10^{-6} M cyclic AMP. The ATP concentration was 3×10^{-3} M. The dose-response curve for the incorporation of ^{32}P into the major peak obtained upon SDS-PAGE of the peripheral proteins ($\circ$) was carried out at a cyclic AMP concentration of 4×10^{-6} M. Errors are $\pm$ s.d., $n=6$ for six different plasma membrane preparations. Similar dose-response curves can be obtained when plotting ^{32}P incorporation into the two other major peaks obtained on SDS-PAGE.

apparently elusive effect on liver⁹, for in the liver, cyclic AMP levels must be raised above their basal levels before insulin can trigger the cyclic AMP-dependent activation and phosphorylation of the cyclic AMP phosphodiesterase (Fig. 2).

Assayed at 1 μM cyclic AMP, the plasma membrane cyclic AMP phosphodiesterase activity of 55 $\text{pmol min}^{-1} \text{mg}^{-1}$ represents only some 10% of the total cellular activity^{2,9}, which one can compare with a basal adenylate cyclase activity of 17 $\text{pmol min}^{-1} \text{mg}^{-1}$ (refs 12, 13). Activation of the phosphodiesterase by insulin doubles its activity, to some 18% of the total, and in the presence of 1 nM glucagon, adenylate cyclase activity increases about fourfold^{11,12}. It has been pointed out that small changes in phosphodiesterase activity could have a considerable effect on cyclic AMP concentration if the turnover rate was high¹⁴ and the stimulation by insulin may take

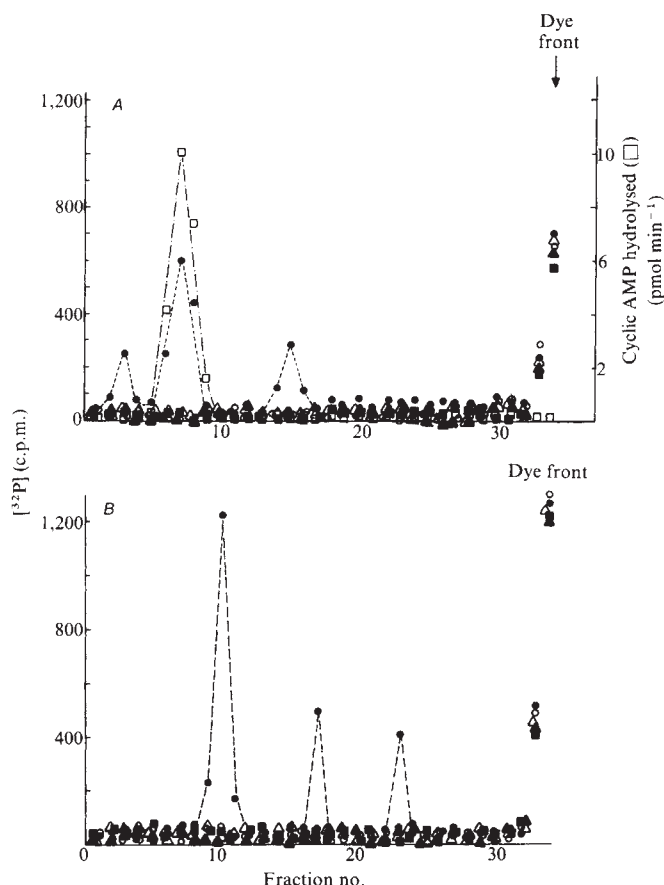


Fig. 3 Polyacrylamide gel electrophoresis of the peripheral proteins of rat liver plasma membranes. **A**, Peripheral proteins were released and subjected to PAGE in non-denaturing conditions with subsequent slicing of the gel as described in ref. 2. This figure demonstrates a typical experiment which we have now carried out over 10 times. Membranes were pretreated with various ligands in the conditions described in the Fig. 1 legend except that $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was present at a final concentration of 1 mM with a specific radioactivity of 200 mCi mmol⁻¹. 100 μg of protein was loaded onto each gel. Gel slices were assayed for ^{32}P after incubation overnight in buffer². Δ , $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ alone; $\blacktriangle$, $[\gamma\text{-}^{32}\text{P}]\text{ATP} + 10^{-4}$ M cyclic AMP; $\circ$, $[\gamma\text{-}^{32}\text{P}]\text{ATP} + 10^{-6}$ M insulin; $\bullet$, $[\gamma\text{-}^{32}\text{P}]\text{ATP} + 10^{-4}$ M cyclic AMP + 10^{-6} M insulin; $\blacksquare$, $[\gamma\text{-}^{32}\text{P}]\text{ATP} + 10^{-4}$ M cyclic AMP + 10^{-6} M insulin + cyclic AMP protein kinase inhibitor protein (4 $\mu\text{g ml}^{-1}$). Where $[\gamma\text{-}^{32}\text{P}]\text{ATP} + 10^{-4}$ M cyclic AMP + 10^{-6} M insulin were used, the eluted gel slices were also assayed for cyclic AMP phosphodiesterase activity². Identical quantities of protein were loaded onto and extracted from each gel, making the results directly comparable. **B**, SDS-PAGE of the peripheral proteins. An identical experiment to that described above was set up, except that 100 μg of protein in 10 μl was mixed with 5 μl of a cocktail containing 6 mM EDTA, 0.75 M sucrose, 6% SDS, 0.03% bromothymol blue at a final pH 7.4. 160 mM dithiothreitol (5 μl) was added and the mixture incubated for 5 min at 100°C. After cooling, 3 μl of 0.5 M iodoacetamide was added and this mixture incubated for 15 min at room temperature. SDS-PAGE was then carried out by the method of Laemmli²⁵ and gels were then sliced and incubated² except that 0.1% SDS was added to the buffer. This figure demonstrates a typical experiment. Similar results were obtained in both instances using 4×10^{-6} M cyclic AMP and 10^{-9} M insulin.

advantage of this. Furthermore, theoretical studies imply an importance of a low K_m phosphodiesterase bound to the plasma membrane in regulating both overall and local cyclic AMP concentrations¹⁵. However, we should not discount the possibility that a considerable fraction of the cyclic AMP phosphodiesterase activity is associated with intracellular membranes² and that the rapid internalization of insulin by endocytosis¹⁶ may lead it to achieve a similar activation there. Furthermore, insulin may depress cyclic AMP levels in part by inhibiting adenylate cyclase in a Ca^{2+} - and adenosine-dependent fashion¹⁷.

Occupancy of the insulin receptor in liver can lead to the cyclic AMP-dependent phosphorylation of three peripheral membrane proteins. One of these is a low K_m cyclic AMP phosphodiesterase which becomes activated. It is intriguing to

speculate whether the other proteins are involved in Ca^{2+} mobilization^{5,18} or in the generation of a 'mediator' of some of the other known intracellular actions of insulin^{19,20} and to what extent intrinsic proteins may be affected. It would seem that, unlike the glucagon receptor where occupancy leads simply to the activation of adenylate cyclase, an occupied insulin receptor may affect several target proteins by initially triggering a membrane-bound cyclic AMP-dependent protein kinase.

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Stimulation of RNA synthesis in isolated nuclei by an insulin-induced factor in liver

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Insulin stimulates the transport of small molecules as well as the synthesis of triglyceride, glycogen, DNA, RNA and protein. Although it is widely accepted that its differing actions are consequent to the binding to surface receptors, there is suggestive evidence that it may also act intracellularly. In 1975, we reported two well defined intracellular insulin binding sites in rat liver: the nucleus and the endoplasmic reticulum¹. Specific binding sites for insulin on intact nuclei were shown by an immunofluorescent and autoradiographic technique, and the kinetics of insulin binding to isolated nuclear membranes were described^{2,3}. Similar results have been obtained by other investigators^{4–6}, and the more recent reports on the internalization of insulin^{7–9} give further support for an intracellular site of action of intact insulin or of its metabolic product. Investigation of the stimulatory effect of insulin on the synthesis of DNA and RNA in diabetic rats and isolated hepatocytes^{10–13}

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suggests that the stimulation of RNA synthesis precedes that of DNA, and is likely to be an early event in the stimulation of macromolecular synthesis. I report here evidence of a low molecular weight substance in rat liver perfused with insulin which can stimulate the synthesis of RNA in isolated nuclei. These results suggest that insulin may function by binding to the cell surface, initiating or enhancing endocytosis¹⁴ and entering the cell via a micropinocytotic route⁹. Once inside the cell, the active principle could be insulin itself, an intracellular degradation product of insulin, or a metabolic product of insulin action.

Fasted male rats were anaesthetized with Nembutal and their livers perfused with the perfusion apparatus described by Miller¹⁵ (MRA Corporation, Clearwater, Florida). The perfusions were performed for between 5 and 30 min at 37°C. The medium was Hank's balanced salt solution 50 mM in HEPES and 3% in bovine serum albumin pH 7.5, with (10^{-7} and 5×10^{-8} M), and without insulin. The perfusate was equilibrated with 95% O₂ and 5% CO₂. Flow rates of 15–20 ml per min were maintained by a constant hydrostatic pressure. In these experimental conditions livers remain viable for 2–3 h without red blood cells in the perfusing medium¹⁶. In some instances the O₂ uptake of the liver was measured. The P_{O_2} between the inflow and outflow was lowered from 450 to 150 mm Hg. The concentration of immunoreactive insulin was determined in the perfusion medium before the onset, and after termination of the perfusion. Between 40 and 50% of the insulin was lost from the medium which is in good agreement with other reports^{17,18}.

After the perfusion was completed, the liver was immediately washed with ice-cold saline, homogenized in 0.25 M sucrose (20 mM in Tris-HCl pH 8.0) and fractionated into three sub-cellular fractions, nuclear, MLP (see below) and cytosol, by differential centrifugation¹⁹. The mixed particulate fraction, MLP (mitochondrial-lysosomal-microsomal), was obtained by centrifugation of the nuclear supernatant at 180,000 g for 1 h. The pellet was washed twice, suspended in 10 ml of 50 mM Tris-HCl buffer pH 7.8, sonicated with the MSE sonifier for 2 min and finally centrifuged again at 180,000 g for 90 min. Identical results were obtained when the vesicles were disrupted by multiple freezing and thawing. The soluble part, MLP soluble, was carefully separated from the pellet and the pellet suspended in 6–7 ml of 50 mM Tris-HCl pH 7.8 (MLP membrane). Both the MLP soluble and cytosol in subsequent steps were fractionated with the use of the Amicon ultrafiltration system. The first filtration was performed with the PM 30 membrane filter. In several experiments the fraction containing cellular components below 30,000 molecular weight was further fractionated with the UM-2 Amicon filter. The final fractions of the respective experiments (molecular weights below 30,000 or below 1,000) were lyophilized, suspended in 1 ml of water and used in the nuclear RNA synthesizing system. Nuclei from young rats (130 g) were isolated and characterized as described earlier³ by the method of Blobel and Potter²⁰. The assay conditions for RNA synthesis are described in the legend to Fig. 1.

Figure 1 depicts the results of a typical experiment indicating that perfusion of rat liver with 5×10^{-8} M insulin for 10 min produced a factor that directly stimulated the incorporation of ³H-UMP into trichloroacetic acid insoluble material by isolated rat liver nuclei. The range of results obtained with different

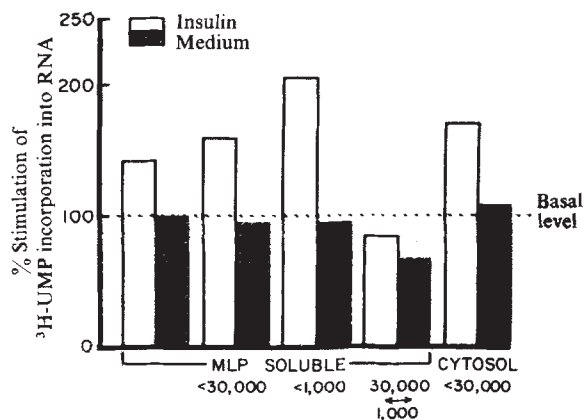


Fig. 1 Effect of insulin-induced factor on RNA synthesis in isolated rat liver nuclei. The livers were perfused with (5×10^{-8} M) or without insulin for 10 min; fractions and nuclei were isolated as described in the text. The assay for RNA synthesis was performed at 30°C in a 250 l incubation mixture which was: ATP 400 μ M, CTP, GTP 200 μ M each, ³H-UTP (NEN, 23.7 Ci mmol⁻¹) 330 nM, MnCl₂ and dithiothreitol 4 mM each, (NH₄)₂SO₄ 250 mM, Tris-HCl 40 mM, pH 7.9. The reaction was started with the addition of an aliquot of the nuclear suspension corresponding to 30–40 μ g of DNA. After 5 min of incubation in a shaking water bath the reaction was stopped with 1 ml of ice-cold 10% trichloroacetic acid (TCA) and after 30 min at 0°C the precipitate was filtered on GF/C membrane filters, washed with 15 ml ice-cold 5% TCA, dried under IR lamp and counted in a scintillation counter with a 55% efficiency. The basal values (100% on the ordinate) correspond to an incorporation of 20 to 25 pmol UMP per mg DNA per 10 min. The amount of various fractions added was between 50 and 100 μ g of protein as determined by the method of Miller²¹ with bovine serum albumin (Armour) as standard.

nuclear preparations and with fractions obtained from five insulin perfused and four non-insulin perfused liver preparations was as follows: insulin: MLP soluble, 138–200%; MLP soluble <30,000, 142–185%; MLP soluble <1,000 148–250%; cytosol <30,000, 124–200%. The corresponding values for the fractions from control livers (no insulin) were: 98–108%; 95–108%; 95–120%; 95–103%; and 96–110% of the basal levels. No stimulation was observed when intact insulin (10^{-8} – 10^{-6} M) was substituted for the perfused liver fractions in incubations with isolated nuclei (three experiments). With the MLP membrane fraction, an inhibition was noticed in two experiments. Both the basal level of RNA synthesis and the degree of stimulation depended greatly on the nuclear preparation, an observation also made by other workers²². Therefore, each nuclear preparation before use was checked for linearity in incorporation of ³H-UMP with time and concentration of nuclei.

The results shown in Fig. 1 were all obtained in assay conditions optimal for RNA polymerase II, that is high [NH₄⁺] and in the absence of α -amanitin. As shown in Table 1, when α -amanitin was added to the incubation mixture at a very low concentration (0.8 μ g ml⁻¹), 75% of the basal activity was inhibited indicating that a large portion of the measured basal activity is due to RNA polymerase II²³. All of the stimulation obtained by the MLP soluble fraction from the insulin perfused liver was completely abolished by the addition of this low concentration of α -amanitin. This could indicate that the stimulator specifically enhanced the synthesis of hnRNA in isolated nuclei²³. The remaining activity is probably due to RNA polymerase I since the addition of 100 μ g ml⁻¹ α -amanitin did not affect the residual incorporation of ³H-UMP into RNA²³.

In another set of experiments a dose-response effect could be demonstrated with fractions containing as much as 100 μ g of protein, the latter amount not consistently exhibiting stimulation above that obtained with 50 μ g. In a few experiments, the livers (insulin perfused and control) were fractionated into separate particulate subcellular fractions: nuclei, mitochondria,

Table 1 Effect of α -amanitin on the stimulation of RNA synthesis by a factor induced in liver perfused with insulin

Medium	³ H-UMP incorporated (d.p.m.)
Basal	21,000
+ α -amanitin	5,200
Basal + MLP soluble	29,000
+ α -amanitin	5,300

The assays were performed as described in the legend to Fig. 1. α -Amanitin concentration 0.8 μ g ml⁻¹, MLP (100 μ g protein) added where indicated.

lysosomes, microsomes each of which was treated as described above for the MLP. Stimulation of RNA synthesis was observed with the lysosomal and to some extent with the microsomal fraction, but not with the mitochondrial or nuclear fraction.

These results suggest that the vacuolar apparatus of the cell is associated with an active principle resulting from exposure of the cell to insulin. The substance is of low molecular weight, 1,000 or less, and conceivably could be a degradation product of insulin. Alternatively, it could be a compound chemically unrelated to insulin. In this regard, reports of a synthetic hexapeptide corresponding to the B₂₁₋₂₆ fragment of insulin having a stimulatory effect on glucose uptake in the hind limb of rats could be pertinent to these results^{24,25}.

Turakulov *et al.*²⁶ reported the partial purification of a cytoplasmic regulator of Ca²⁺ transport by mitochondria isolated from rat liver 45 min after an intraperitoneal injection of insulin. These authors define the cytoplasm as the 30,000 g supernatant of the homogenate which actually contains both lysosomes and microsomes. From their results it seems that the factor is present in free and bound form supporting the suggestion presented in this paper, that is, that the primary site of formation of the active principle are membrane bound vesicles. The stimulation of RNA synthesis observed by the cytosol does not invalidate this suggestion since lysosomes are very fragile organelles and easily disrupted even with the most careful homogenization. Insulin has a delayed effect on the stimulation of RNA synthesis as compared to its very rapid effect on transport. If the formation of the active principle is indeed taking place in the vacuolar apparatus of the cell, and if it is a degradation product of insulin, the delay can be explained by the time needed for the content of the vesicles to be released into the cytoplasm. In general it is considered that the lysosomal membrane is freely permeable to small molecules, although it was also shown that 2 hours after the injection of ¹⁴C-sucrose into the rat, subsequently isolated lysosomes contained both ¹⁴C-glucose and ¹⁴C-fructose²⁷. As the factor appears to be of molecular weight below 1,000 it could reach the cytoplasm and finally the nucleus either by diffusion from the secondary lysosomes or alternatively by pyrolysis, a process by which material entering the cell via endocytosis can be released into the cytoplasm of the cell without necessarily being completely degraded²⁸.

Recently, a low-molecular weight 'insulin mediator', probably a peptide, was reported to be present in insulin-treated rat muscle²⁹. It mimics the action of insulin on cyclic AMP-dependent protein kinase, phosphoprotein phosphatase²⁹ and pyruvate dehydrogenase³⁰ in a cell-free system. There are several similarities between the 'mediator' and the factor detected in the present study. Both appear after a short exposure of the tissue to insulin, are of low molecular weight and have insulin-like action in a subcellular system, contrasting with previous experience where the action of insulin could be observed only in the whole animal, organ, or at least in the intact cell. There have been no previous reports of any effect of insulin or any compound derived from its action on the stimulation of RNA synthesis in a cell-free system. This report indicates the formation of a compound acting directly on the genetic material. As the insulin-induced factor enhances the synthesis of mRNA precursors it is possible that the factor affects the expression of genes known to be under the control of insulin³¹⁻³⁴.

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Sequencing of 16S-23S spacer in a ribosomal RNA operon of *Euglena gracilis* chloroplast DNA reveals two tRNA genes

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Chloroplasts of the unicellular flagellate eukaryote *Euglena gracilis* contain several copies of a circular 135-140-kilobase pair DNA¹ which codes for chloroplast-specific stable RNAs (16S, 23S (refs 2, 3), 5S rRNAs⁴ and tRNAs⁵) and for an unknown number of chloroplast-specific proteins. The rRNA genes are located within three tandemly repeated DNA regions of approximately 5.6 kilobase pairs each⁶⁻⁸ and the arrangement of the structural genes within each repeat follows the prokaryotic pattern, being 5'-16S-23S-5S-3' (ref. 9). Total chloroplast tRNA hybridizes to fragments of rDNA⁹ and it was suggested that the 16S-23S spacer region contains tRNA coding sequences as is observed in *Escherichia coli*^{10,11} and in spinach chloroplast¹² rDNA. We have therefore analysed *E. gracilis* strain Z 16S-23S spacer DNA at the nucleotide level, hoping this would allow identification of tRNA genes together with the processing sites of the respective primary transcripts. Maize chloroplast 16S rDNA shows strong sequence homology with *E. coli* 16S rRNA¹³. Sequence analysis of a total spacer in *E. gracilis* should demonstrate whether such similarities are also preserved in the chloroplast rDNA spacer region, or if this region has suffered a higher genetic drift rate. The latter is suggested from the 189 bases which have been sequenced from the 2.4-kilobase pair rDNA spacer from maize chloroplasts¹⁴. Flanking sequences, coding for the 3'-terminal region of 16S rRNA and for the 5'-terminal region of 23S rRNA have also been sequenced, to compare the drift rates between the spacer and its adjacent structural genes.

Fig. 1 *a*, Position of the *E. gracilis* rDNA region and of the spacer-containing fragment *Bgl*II-*Hpa*I_{1,500} within the cloned fragment *Bam*HI-D (pEgc11)⁸. *b*, Fine mapping of fragment *Bgl*II-*Hpa*I_{1,500} using the enzymes *Hae*III, *Hpa*II and *Hinf*I. *c*, Fragments used for sequence analysis of the spacer region. The arrows represent portions of 5'-terminally labelled single fragments from which unambiguous sequences could be obtained by a series of overlapping gels. Nearly all positions of fragment *Hae*III₂₂₅ have been analysed from both strands. Sequences of the right-hand portion of fragment *Bgl*II-*Hae*III₆₅₀ could be confirmed by analysing part of the complementary strand of fragment *Hinf*I₄₃₀, which also gives the necessary overlap between fragments *Bgl*II-*Hpa*I₆₅₀ and *Hae*III₂₂₅. Positions of rRNA and tRNA coding sequences and of the intergenic regions A, B and C are indicated. □, *Bam*HI; ▽, *Hpa*I; △, *Hae*III; ○, *Bgl*II; ●, *Hpa*II; ▲, *Hinf*I.

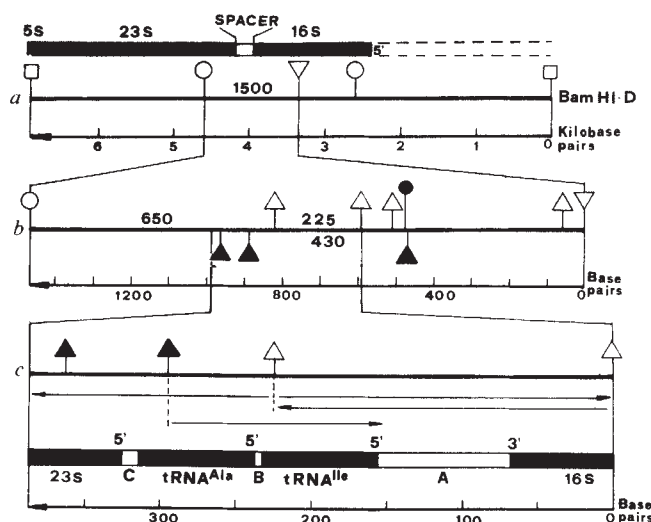


Figure 1 shows the protocol which was followed for mapping and identification of the spacer region within the restriction fragment *Bam*HI-D. This fragment, which contains one of the three rDNA repeats, has previously been cloned using the plasmid pBR322 as vector¹⁵ and the respective clone pEgc11 (formerly pEgcKS11) was used for isolation of fragment *Bam*HI-D and its subfragments. Partial sequencing of several of the subfragments¹⁶ revealed strong homology with regions from *E. coli*^{17,18} and maize chloroplast¹³ 16S rRNA (rDNA) and thereby allowed location of the 3'-terminal region of the 16S rRNA gene on the right-hand third of fragment *Hae*III₂₂₅. Since hybridization and fine-mapping data had indicated^{6,7,19} that the spacer should not exceed 300 base pairs, the left-hand two-thirds of fragment *Hae*III₂₂₅ together with the neighbouring portion of fragment *Bgl*II-*Hae*III₆₅₀ should comprise the entire spacer sequence. These fragments were therefore chosen for sequence analysis according to the dimethylsulphate/hydrazine procedure²⁰ and the necessary overlap between the two *Hae*III fragments was obtained by sequencing the left-hand portion of fragment *Hinf*I₄₃₀.

In Fig. 2 the RNA-like strand of the *E. gracilis* spacer DNA sequence is depicted alongside the respective sequences from

the *E. coli* *rnd* operon. The first 63 positions comprise the 3'-terminal region of the 16S rRNA gene, the 'prokaryotic character' of which is documented by its high degree of homology with the corresponding rDNA region from *E. coli*. The reported 3'-terminal oligonucleotide AACAAUCUN of *E. gracilis* 16S rRNA²¹ allows exact positioning of the 3'-terminal end of the structural part of this gene. The reported T₁ oligonucleotide UAACAAG²¹ which is highly conserved in prokaryotic 16S rRNA²² is found at position 22-28. The non-conserved position 59 is of particular interest, as it partly changes the recognition sequence for translational initiator regions^{23,24}. This suggests that *E. gracilis* chloroplast ribosomes have a slightly altered pattern of recognition for mRNA in comparison with *E. coli* whereas in maize chloroplast ribosomes the corresponding sequence is identical to the *E. coli* 16S rRNA 3' end¹⁴.

A stretch of 66 nucleotides shown in the lower part of Fig. 2 represents the 5'-terminal portion of the 23S rRNA gene as evidenced by its extensive homology with the 5'-terminal region of *E. coli* 23S rDNA^{10,11}. However, since the 5'-terminal oligonucleotides of *E. gracilis* 23S rRNA are not known, exact positioning of the 5' terminus of the 23S rRNA gene is not

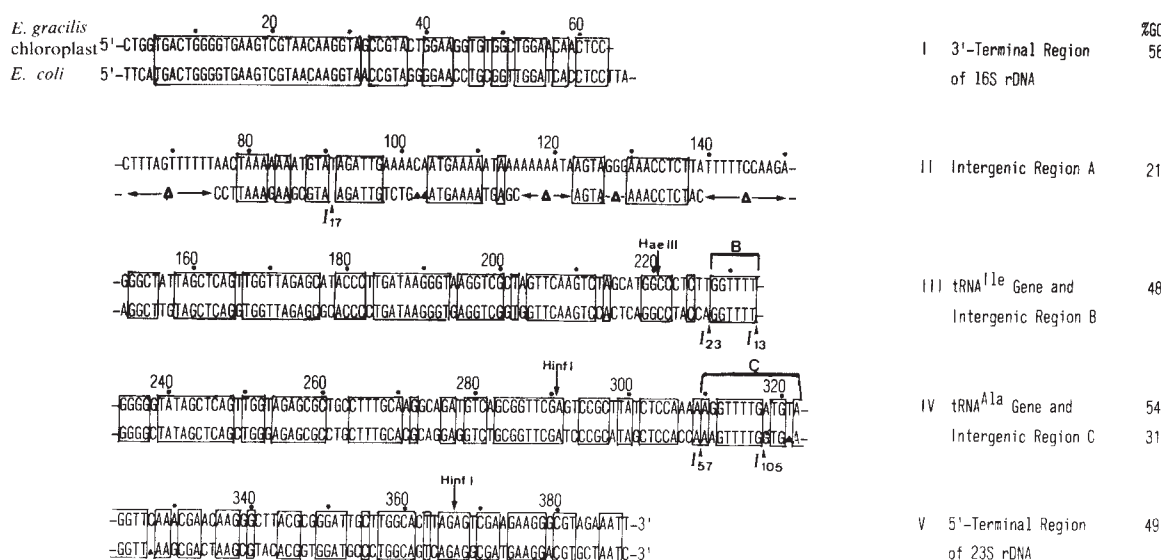


Fig. 2 Nucleotide sequence of the 16S-23S intergenic spacer region and of the flanking region of the 16S and 23S rRNA genes from *E. gracilis* chloroplast DNA (top row) and from the *rrnD* operon of *E. coli*^{10,11}. Only the sequences of the RNA-like strands are depicted. Homologous sequences are marked by boxing. Within the *E. coli* sequence the symbols ▲, △ and I indicate single point deletions, multiple deletions and insertions in respect to the *E. gracilis* sequence. The number *a* of inserted bases is indicated by *I_a*. Note that the *E. gracilis* sequence is depicted in the opposite orientation from that of Fig. 1. Cleavage sites for the restriction enzymes used in Fig. 1 are indicated.

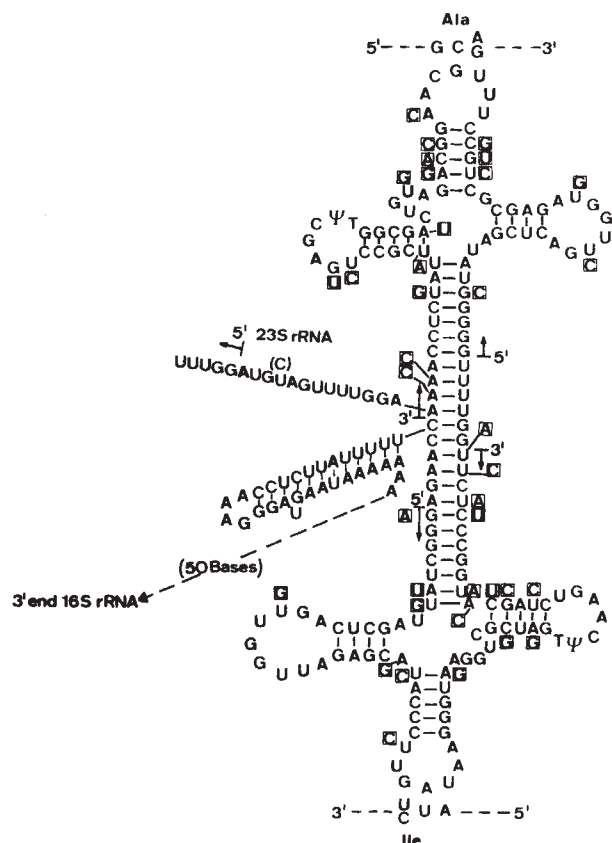


Fig. 3 Possible secondary structure of the dimeric tRNA precursor region as predicted from the corresponding DNA sequence of Fig. 2. The two tRNA cloverleaf structures are depicted without modifications of the bases except for the T Ψ sequences in the T-arms. Bases different in the respective *E. coli* tRNA species^{10,11} are indicated within boxes.

possible. The 16S–23S spacer DNA is thus represented by the 259 base pairs confined within positions 63 and (probably) 322. The analogous region in the *E. coli* rrnD operon is considerably larger with 437 nucleotide positions. In *E. coli* the spacer region is known to be transcribed as a part of the single transcriptional unit of the rrnD operon¹⁰. A large rRNA precursor containing both the 16S and 23S rRNA was also observed in spinach^{25,26} and *Chlamydomonas*²⁷ chloroplasts. A rRNA of similar large size has been observed also in *E. gracilis* chloroplasts²⁸ and therefore it is reasonable to assume that a similar situation exists in this case although direct experimental evidence for transcription of the intergenic spacer is lacking.

Screening for GTTC sequences, which are indicative for the GT Ψ C sequence present in the Ψ loop of all tRNA species²⁹, and for homology with the *E. coli* rDNA spacer region reveals two tRNA genes coding for an isoleucine (AU Ψ)- and an alanine (GC Ψ)-accepting species, respectively. The tRNA primary structures deduced from the two tRNA genes allow folding to a cloverleaf model completely consistent with all other structural criteria of tRNAs²⁹ (see Fig. 3). The deduced cloverleaf models contain several compensating base changes in the stem regions in addition to changes in the unpaired regions, which excludes the possibility that the two genes are derivatives of the *E. coli* genome due to a cloning or recombinational artefact. Also, the two chloroplast tRNA genes lack the CCA termini found in the respective *E. coli* tRNA genes^{10,11}. The two tRNA genes are separated from each other by only six base pairs, which is reminiscent of the clustering of tRNA genes on the phage T $_4$ genome³⁰. Within the precursor transcript, the two acceptor stems together with their neighbouring sequences can form a continuous stack of 24 base pairs (plus 2 $\times$ 5 base pairs of the two T-stems according to the tRNA tertiary model^{31,32}) as drawn in

Fig. 3. A possible hairpin structure directly preceding the 5' end of the tRNA^{Ile} is also depicted in Fig. 3, since its stem region could possibly also form an uninterrupted stack with the acceptor stem (and the T-stem^{31,32}) of this tRNA within the precursor molecule. It is tempting to speculate that some of these possible secondary structures constitute at least part of the signal structures necessary for proper processing of the common precursor chain. Alternatively, or in addition to this, recognition of the sequence GGUUUUG, which is common to both the tRNA precursors immediately beyond the 3' ends, may act as a signalling step during the processing events.

A comparison of the rRNA and tRNA coding sequences with the intergenic regions (Fig. 2) shows that the former are highly conserved contrary to the latter which are subject to many alterations, mainly by deletions but including some base substitutions. The short homologous stretches in the intergenic regions may have some functional significance for processing steps. Furthermore it is interesting that, similar to *E. coli* rDNA^{10,11}, the G + C content is high in the structural and low in the intergenic parts.

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Erratum

In the letter 'Juvenile hormone mimics the photoperiodic apterization of the alate gynopara of aphid, *Aphis fabae*' by Jim Hardie, *Nature* **286**, 602–604, line 2 of paragraph 7 should read 'juvenile characters in the fifth instar. After scoring these alates' and line 5 of the same paragraph should read 'juvenilization are the second and third instars, an observation'.

MATTERS ARISING

The rhenium–osmium age of the Galaxy

LUCK, BIRCK AND ALLEGRE¹ have reported new meteoritic data on Re and Os which led to limits to the age of the Galaxy of between 13,000 and 22,400 Myr. Here I point out how these results change if one uses estimates of the s-process contribution to ¹⁸⁷Os given by Woosley and Fowler².

The isotope ratio used as a galactic chronometer can be written

$$R \equiv \frac{{}^{187}\text{Os}^*}{{}^{187}\text{Re}} = \frac{{}^{187}\text{Os}^*}{{}^{186}\text{Os}} \bigg/ \frac{{}^{187}\text{Re}}{{}^{186}\text{Os}}$$

where all values are for the meteorites at formation and ¹⁸⁷Os* is the radiogenic component of ¹⁸⁷Os. The s-process component is written in terms of the ratio of neutron capture cross-sections and a correction factor, *f*, so that

$${}^{187}\text{Os}^*/{}^{186}\text{Os} = ({}^{187}\text{Os}/{}^{186}\text{Os})_{\text{total}} - (\sigma_{186}/\sigma_{187})f$$

Let us use from reference 1 the values ¹⁸⁷Re/¹⁸⁶Os = 3.20 and (¹⁸⁷Os/¹⁸⁶Os)_{total} = 0.805 ± 0.011 (2σ), and from ref. 2 the values σ₁₈₆/σ₁₈₇ = 0.504 ± 0.034 (2σ) (ref. 4) and 0.8 ≤ *f* ≤ 1.15. These quantities give *R* = 0.096 ± 0.041. Luck *et al.*¹ use a smaller s-process contribution, which gives *R* = 0.152.

A firm lower limit to the age of the Galaxy comes from assuming that all nucleosynthesis of Re occurred in a single burst at time *t* = 0. Then the time *T* of meteorite formation (4,550 Myr ago¹) is given by *R* = exp(λ*T*) − 1, where the decay constant λ = 1.62 × 10^{−11} yr^{−1} (ref. 1); the age of the Galaxy is given by *t*₀ > *T* + 4,550 Myr. The value of *R* used by Luck *et al.* gives *t*₀ > 13,300 Myr, but the above revised value gives a lower limit of 10,200 ± 2,300 Myr.

An upper limit to *t*₀ cannot be obtained without a detailed model of chemical evolution, including gas flows³. As a simple example of an older model, the case stated by Luck *et al.* of 'steady-state nucleosynthesis' gave *t*₀ = 22,400 Myr with their value of *R*, but with the above value it gives *t*₀ = 16,100 ± 5,100 Myr.

The main points are, as foreseen by Woosley and Fowler², that uncertainties in the s-process seriously affect the Re–Os chronometer, and that use of the latest estimate of the s-process contribution to ¹⁸⁷Os reduces the lower limit to the age of the Galaxy by about 3,000 Myr.

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Accuracy of thermoluminescence dates

WE congratulate Liritzis and Thomas¹ on the first combined application of thermoluminescence dating and magnetic palaeointensity determination, particularly in view of the disadvantageous magnetic characteristics of the kiln wall material used. It is a pity, however, that the thermoluminescence age errors in their Table 1 have been quoted without qualification, as they are liable to mislead archaeologists and others into expecting a higher accuracy from the technique than it can truly give. As has been learnt with radiocarbon dating, it is important to distinguish between precision of measurement and absolute accuracy. Evidently the errors quoted in Table 1 refer to the former (because the errors quoted for the averages are substantially less than the errors quoted for the individual measurements).

The systematic errors inherent in thermoluminescence dating are of a different type from those in radiocarbon but they are, nonetheless, important if comparison is being made with archaeologists' calendar year dates. Whereas the major systematic errors in radiocarbon dates have been worldwide effects—initially, uncertainty about the value of the half life and, more recently, uncertainty about the calibration curve by which radiocarbon years are converted into calendar years—the systematic errors in thermoluminescence dating arise from more intimate effects such as differences between types of fabric measured, burial environments and laboratory techniques.

It is not appropriate here to go into detail, but on the basis of a previous assessment^{2,3} and taking into account two additional interfering effects also noted^{4,5} specifically in respect of quartz, it seems unlikely that tighter limits than ±5% can at present be placed on the possible systematic error for a thermoluminescence date. This is at the 67% level of confidence. Neglecting any contribution from measurement error, this 5% systematic error limit corresponds to ±200 yr for a date at around 2000 BC, falling to ±150 yr for 1000 BC. More realistically, we would put the best overall error limit that can be achieved for the average date on a group of contemporaneous samples, except in abnormally favourable circumstances, at ±7%, that is, ±300 yr and ±200 yr, respectively.

Obviously it is to be hoped that research will lead to improvement in the accuracy of thermoluminescence dating. One notable obstacle is the uncertainty introduced by possible variation in water content during burial; although techniques have been proposed^{6,7} that eliminate this interference, they have not yet reached a better accuracy than that quoted above.

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LIRITZIS REPLIES—I would like to clarify some points. First, the systematic errors in thermoluminescence (TL) dating of well-fired kilns clays are not pronounced (as they are in most other cases), due to the kiln structure; we used the internal part of the well-fired kiln wall or internal part of a fused kiln-surface clay. Second, the dated quartz samples (15 kiln clays and 3 tiles) come from the same fabric type of the respective kiln, and all have been subjected to the same climatological factors, an 'advantage' for precise water uptake and environmental γ-radiation measurements. This mineral similarity was also found from X-ray fluorescence analysis of many samples from the same kiln, and from the TL glow curves.

Third, in general, I agree that the systematic errors of ±5% exist for TL dating, but in the favourable circumstances of studying kiln materials they are much reduced. Fourth, the quoted 67% level of confidence will ensure improved accuracy for archaeologists' estimations. Fifth, some estimated errors (many due to the operator and technique used) cancel themselves out. Therefore, an average date for many contemporaneous samples, in the same burial conditions and weathering as in kiln materials, does make sense and justifiably reduces the overall error. Finally, by taking the 'permitted' average error of many TL dates as an overall error limit of the age of a kiln, the final quoted TL accuracy, as estimated % error, of our report is valid, and the precision obviously better.

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Ignimbrite veneer deposits or pyroclastic surge deposits?

RECENTLY, Walker and others¹ applied the concept of aspect ratio^{2,3} to ignimbrites and we accept this as valid reasoning. An outstanding feature of the low-aspect ratio Taupo and Rabaul ignimbrites (deposits of pumiceous pyroclastic flows) they describe is a landscape-mantling deposit (ignimbrite veneer deposit) which can be traced into a more conventional topography-infilling ignimbrite (valley-ponded ignimbrite). According to Walker and others¹, recognition of these new features requires that a reappraisal be made of the emplacement mechanisms and new diagnostic criteria be drawn up for recognizing pyroclastic types. We, however, question this statement.

In the lesser Antilles, several pyroclastic flow deposits that fill valleys have been traced into finer-grained, stratified and cross-stratified deposits which mantle the interfluvies between valleys. These deposits are interpreted by us as the deposits of ash-cloud pyroclastic surges. Ash-cloud surges are outwardly expanding dilute pyroclastic flows derived from an underflow or pyroclastic flow *sensu stricto*⁴⁻⁶. The flow and surge have fundamentally different transport mechanisms. Pyroclastic flows are high-concentration flows that move by laminar and/or plug flow^{7,8}. Deposits are typically massive, poorly sorted and topographically controlled. Surges are flows that are turbulent and deposit beds with internal stratification and cross-stratification⁴⁻⁶. Surges are not topographically controlled and deposits can mantle landscape adhering to relatively steep slopes.

Walker and others¹ imply that the ignimbrite veneer deposit is the deposit of a high-concentration flow. The veneer is regarded by them as a 'tail deposit' left in the wake of the pyroclastic flow. From their description we contend that this tail deposit is the deposit of an ash-cloud surge derived from the main flow and is its lateral equivalent.

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WALKER ET AL. REPLY—We accept without reservation the relationships described by Fisher and others from the Lesser Antilles. We have long believed in the concept that pyroclastic flows *sensu stricto* and pyroclastic surges have fundamentally different transport mechanisms. What is in question, however, is not the basic concept but the origin of certain rocks occurring specifically at Taupo and Rabaul Volcanoes: the rocks which we have called ignimbrite veneer deposits (IVDs).

We considered very carefully the possibility that our IVDs might have been emplaced in the way that Fisher and others propose, but rejected it for the five reasons outlined below.

First, apart from having a generally smaller content of coarse pumice, the IVD is identical in its grain-size characteristics with the valley pond ignimbrite, and has identical pumice density and crystal and lithic sizes and contents. There is no laboratory test that we can apply to distinguish between the two. Moreover, the IVD is visibly in lateral continuity with, and passes gradually into, the valley pond ignimbrite.

Second, the sheer scale of the IVD at Taupo seems to us to negate the idea that it could be the deposit of a surge generated at the vent; we think it highly unlikely that any pyroclastic surge is capable of moving radially outwards against air resistance, and of remaining highly turbulent long enough, to encompass a nearly-circular area measuring more than 160 km across, or of carrying $>10^{13}$ kg of poorly sorted pyroclastic material held entrained in its gas. We note that all known far-reaching pyroclastic surges have originated on steep and high cones, and the most far-reaching one (Bezmyanny 1956¹) was strongly directional, yet it travelled no further than 29 km. The Taupo IVD is not directional, and there is no evidence that it originated on a steep or high volcanic cone.

Third, the scale again seems to us to preclude the possibility that the IVD was generated by a whole succession of pyroclastic surges jetting forwards from the advancing ash-flow, which is presumably the mechanism that Fisher and others envisage. We do not deny that such jetting may and probably does occur, but we would expect the surges to carry much less particulate material than the parent body. This relationship certainly holds at Mt Pelée (Martinique) where one of us (G.P.L.W.) has worked. At Taupo, however, the IVD constitutes about 50% of the total ignimbrite volume, and at Rabaul as much as 75%. We note that the higher the proportion of 'surge-deposited' material, the more closely in effect does the 'jetting surge' concept converge on the single 'vent-generated-surge' one.

In the 'jetting surge' model we would, moreover, expect some kind of close relationship to exist between the thickness

and lateral spread of a surge deposit and the position of adjacent valley ponds, but we find no such relationship. There are many places where a normal thickness of IVD is found several kilometres laterally from, and often several hundred metres higher than, the nearest substantial valley pond.

Fourth, at Taupo we have found ponded ignimbrite in many valleys where, to reach them, the parent flow must have crossed ridges or passes standing hundreds of metres higher than the present vent position. The most extreme ignimbrite pond we know is 2–3 m deep, contains pumice fragments up to 30 cm in diameter and occurs high on Tongariro Mountain, 45 km laterally from and 1,500 m higher than its source vent. We know of no way that this valley pond could have formed other than by the passage of a high-concentration pyroclastic flow right across the mountain.

Fifth, undoubted pyroclastic surge deposits differ notably in grain size and constitution from ignimbrite. The deposits of a more or less cold (and often wet) base surge are generally well stratified, and the beds (which have a thickness commonly measured in millimetres or centimetres) associated in the same bedding set commonly vary in median diameter over a range of 5 phi units or more. In contrast, in the IVD the layers (apart from certain pumice lenses) vary typically over a range of less than 1 phi, and the presence of carbonized vegetation indicates that they were hot. The deposits of a hot pyroclastic surge (such as the dilute component of a *peléan nuée ardente*) are totally different in character from ignimbrite, typically being very strongly enriched in the denser constituents (lithic fragments, crystals) and strongly depleted in fine dust.

We are convinced from these relationships that our IVD is indeed the deposit from a high-concentration flow, a pyroclastic flow *sensu stricto*, and cannot have been produced by any surge mechanism involving a dilute 'ash cloud'. Clearly, emplacements of both the Taupo and Rabaul ignimbrites were quite exceptionally violent events, far beyond the experience of civilized man, and we believe that there is a limit to how far their products can be interpreted from the study of such relatively minor eruptions as the 1902 *nuée ardente* of Mt Pelée.

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BOOK REVIEWS

Mother science and prodigal sons: a history of haematology

William S. Beck

IN THE discharge of editorial duties one day, I received a critique that included the phrase "This monograph fills a much needed gap". That addled expression came to mind when I contemplated this handsome new book by Maxwell M. Wintrobe and a well chosen gallery of distinguished haematologists. There was indeed a gap — much needed if it inspired this book — in the literature of scientific history. That gap, more like a chasm, concerned haematology, a discipline that richly deserves to have its story told.

The field of haematology has several notable properties. Because blood has always fired the human imagination, it long interested philosophers and poets who contributed some of our earliest notions of it. By drawing the book's title from the emotional imagery of John Donne, Wintrobe reminds us of that fact. Because blood is readily available, it was also an early object of scientific investigation, yielding many important basic discoveries in which haematologists have taken a proper pride. Some were of such magnitude they significantly advanced whole branches of science. There can be no doubt, for example, that the discoveries of liver therapy for pernicious anaemia by Minot and Murphy in 1925 and of intrinsic factor by Castle in 1929 turned haematology from a musty descriptive discipline, dominated by blood cell morphology, into a dynamic science rooted in physiology and biochemistry. These advances launched the whole field of modern clinical investigation. For another example, the discovery that sickle cell anaemia is a 'molecular disease' opened an important new chapter in molecular biology.

These successes had interesting consequences for the mother science, which soon spawned a cluster of sub-disciplines. Like prodigal sons, they achieved their own bountiful successes and left home. Those who would define haematology as the science of blood saw the perimeter breached again and again, as such subdivisions as oncohaematology, immunohaematology and haemostasis acquired their own journals, national societies, sections at the National Institutes of Health and other perquisites. This disconcerting situation is burdensome for all

Blood, Pure and Eloquent: A Story of Discovery, of People, and of Ideas. Edited by M. M. Wintrobe. Pp.771. (McGraw-Hill: 1980.) \$30, £18.50.

who teach haematology to medical students, who must take certifying examinations in medicine or pathology, who plan and fund research programmes, and who apply these disciplines in the care of patients. Still, though it has been something of a nightmare for intellectual taxonomists, haematology has been a raging success scientifically. Its opulent history is dealt with adequately for the first time in this volume. It is a grand homecoming.

Wintrobe, a widely respected academic haematologist, has collected a series of articles on the several haematological subdivisions by recognized authorities, most of whom have themselves contributed significantly to the historical record. The book begins with an essay by Wintrobe that spans the early beginnings, from the ancient roots of haematology, through the great age of microscopy, up to the modern era. Next, an interesting chapter by Hart on palaeohaematology summarizes what can be gleaned about blood in ancient times from contemporary studies of bones, bodies and ancient art — an unusual topic for a treatise on history. There follow chapters on the bone marrow by Tavassoli, stem cells by Lajtha, the spleen by Crosby, red cell metabolism by Beutler, iron and haem by London, haemolysis by Dacie, effects of altitude on blood by Erslev, the conquest of pernicious anaemia by Castle, sickle cell anaemia by Conley, the molecular basis of thalassaemia by Weatherall, phagocytosis and granulocytes by Craddock, lymphocytes by Ford, leukaemia and lymphoma by Gunz, platelets by Spaet, plasma proteins by Janeway, haemostasis by Ratnoff, and blood transfusion and blood groups by Diamond. In a rather brief final chapter, Wintrobe draws the lessons of history.

Certain of the book's features merit commendation. It has a lively literary style and a gratifying uniformity of content,

level and accent. Even so, some of the authors' personalities manage to shine through discreetly. Wintrobe wisely chose to steer between the extremes of readership. It should readily appeal to intelligent readers who may lack a rigorous scientific background but who are willing to make use of the book's convenient glossary. One hopes these will include legislators and other public officials. Yet it is detailed enough to be read profitably by haematologists and serious students. Indeed I can attest that in a recent course medical students found some of the essays a better guide to complex topics than conventional textbooks. This level adjustment is more successful in some parts than in others, but on the whole it has been well done.

Why write a history of haematology — or of any branch of science? Philosophers have long debated the purposes of historical scholarship. Is it the historian's purpose to be 'descriptive', to recount past events for their own sake or for the sake of making a credit-allocating record, or is it his purpose to be 'theoretical', to interpret, to generalize and to seek wisdom that might solve contemporary problems? Or is it simply to interest and amuse?

My only criticism of this attractive book — so lovingly compiled with its fine portraits and useful bibliographies — stems from private notions on historical writing. Most of the historian's purposes are meritorious, but it is possible to pursue them in different ways. One can recount what happened — the dates, names and facts — or one can tell what *really* happened in a sort of *cinéma vérité* style of reportage that conveys the important and fascinating details, never given in ordinary literature, behind the development of scientific ideas — the accidents, personalities, conflicts and inspirations that underlie every major discovery. In my opinion, writing in the latter mode is far more likely to achieve any of the possible purposes of a historian. I am especially impressed by several recent books that reached new levels of candour in the reporting of incidents, personal characteristics and idiosyncracies — books that seemed to tell it as it was. It is a much to be admired trend in the writing of scientific history. Alas, that trend is not greatly

extended by this volume, which has a somewhat conservative tone.

The book's essayists are leaders who have contributed importantly to their own specialties. The editor tells us he chose them for that reason. But the essays are filled with the works of others and barren of the kind of information that one might relish from an important actor on the stage of history, the kind of information that only he possesses. In a felicitous chapter on



Georges Hayem (1841–1935), one of the 'fathers of haematology'.

"blood and mountains" Erslev refers to his discovery of erythropoietin in one sentence in the third person. Why did he do this experiment? How did he do it? There is no one better qualified to tell us than Erslev himself. Beutler deserves praise for having interviewed several major figures in preparing his chapter but he omits to explain how his own work on glucose-6-phosphate dehydrogenase deficiency anticipated what is now called the Lyon hypothesis. London tells us a bit about the Schoenheimer school at Columbia but not enough about his own perceptions of it. Castle speaks interestingly of Minot's personality and antecedents but is a shadowy figure in his third person references to himself. Spaet does not mention his own work. Such modesty!

Of course, this is probably not fair comment because it suggests that the editor and authors should have written a different book. In fact, this book is splendid and highly recommended. My only quibble is that ample room exists for more effort in this area. We all should hope that Professor Wintrobe continues to explore the many trails he has blazed with this welcome work. □

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Russian holography

P.W. Hawkes

Holography and Coherent Optics. By L.M. Soroko. Pp.818. (Plenum: 1980.) \$59.50, £37.49.

THE decision to publish this translation, of a Russian text which first appeared in 1971, is most astonishing. At that date it was remarkably up to date, but holography is surely one branch of applied physics that has progressed out of all recognition during the 1970s. Further, the book lacks the merit of bringing early Russian work in this field to the attention of Western eyes, for there are no references at all. To cap this, it has the defect common to many Russian scientific texts: there is no index.

The book opens with some general ideas about holography, after which three chapters deal with various transforms of

particular importance in optics, random signals and optical coherence. A chapter is then devoted to information theory in optics, followed by one on holography. The Russian text then concluded with a discussion of optical information processing, but for the English edition, the 'translation editor', G.W. Stroke, has included three of his own publications. Each topic is dealt with at some length, but for almost every subject much progress has been made since the book was written. In short, there really was not much point in translating this text, for in so fast-moving a subject students and research workers of the 1980s will need more recent information than that collected here. Access to a set of Professor Wolf's annual *Progress in Optics* (North-Holland) would be a great deal more useful. □

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More of the Amboseli baboons

John M. Deag

Baboon Mothers and Infants. By Jeanne Altmann. Pp. 242. (Harvard University Press: 1980.) \$17.20, £10.50.

EVER since the publication of *Baboon Ecology* by S.A. Altmann and J. Altmann (University of Chicago Press: 1970), primatologists have been waiting for further information on the social behaviour of the Amboseli baboons. *Baboon Ecology* set a new standard for primate ecological studies and Jeanne Altmann's *Baboon Mothers and Infants* will have equal influence on research into social relationships. This is no ordinary report of mother-infant behaviour and those most familiar with laboratory studies will find its scope unusual. Altmann summarizes her aims as follows: "... to assess the nature and extent of external influences on baboon mothers and their young infants. My general goal was to identify and measure factors affecting survival and behavior during motherhood and infancy, and to identify likely ontogenetic origins of differences in adult behaviour and life history patterns". The book therefore represents one of the first serious attempts to portray the total behavioural and ecological relationships among mothers, infants and the social group of which they form such a fundamental part.

It starts with an introduction to baboons and their habitat, and a short but important section on demography. During the first two years of life there was a per

annum mortality of 0.3. Several infants were ill, wounded or suffering from possible nutritional deficiencies or poor mothering, and the author uses such cases when considering the costs and benefits of different sorts of behaviour. Mothers with young are themselves vulnerable, and older females with dependent young were found to be particularly at risk of dying when compared with younger females or those in other stages of the reproductive cycle. The effects of infants on their mothers' feeding patterns and time budgets are described and formalized in a theoretical model. It seems that if a mother had to provide all her infant's nutrition, severe time-budget problems would occur with infants more than 6–8 months old, even at slow rates of infant growth. A mother simply cannot gather enough food to maintain her body weight and to feed her infant; weaning is consequently forced upon the pair. Later sections of the book deal more directly with behavioural topics such as maternal care, infant development, spatial relationships, weaning and independence, and with how a mother's social relationships are affected by her infant's age.

The study group has been under observation since 1971 and a great deal of background information is available. The quantitative results on mothers and infants in the present study are drawn from 15 months of field work and 18 infants. Sound quantitative methods are used to provide an extremely detailed picture of the infants and their mothers. The major strength of this book lies in the detailed analyses attempted; but this is paradoxically also its main weakness, since it may make much of the book of little interest, except to the specialist prepared to wade through all the intimate details. The necessity of referring to individuals is a problem faced by all who

deal in complex social relationships and yet who, except the author and her co-workers, can find it easy to follow a text studded with names which need constant back-reference to determine age, social rank and sometimes even sex? Such diversions are distracting but it is merely the presentation which is at fault — the philosophy of looking at individuals in detail is sound. No proper understanding of primate social behaviour and its evolution will be developed unless we have robust information on individual differences: on this count the book makes an important contribution. Although the sample of 18 infants may sound small it is in fact large by field (and even some laboratory) standards.

One of the major challenges of primatology is to see through the tangle of individual differences to reveal valid generalizations and thereby derive an understanding of the basic principles governing individual behaviour. As students of human personality will no doubt point out, this is not a straightforward task. Altmann searches for such generalizations wherever her sample size permits, and finds, for example, some interesting relationships between a mother's rank and her interaction with her infant and other group members. As would have been expected, low-ranking mothers gave more distress responses (submissive behaviour patterns) during social interactions and their infants were particularly prone to being interfered with by other members of the group. Mothers of different social rank also tended to show different styles of mothering: high rankers tended to be '*laissez-faire*' mothers (mothers tolerating separation from their infants); low rankers tended to be 'restrictive' mothers (mothers maintaining close contact with their infants). These



Jeanne Altmann's 'Alto's Group' of baboons, resting and grooming in Amboseli National Park, Kenya.

differences in mothering appear to have consequences for infant maturation — infants of *laissez-faire* mothers became independent more rapidly. However it is much easier to describe such behavioural differences than to assess the costs and benefits (in terms of genetic fitness) with which the individual differences in behaviour are associated. The author recognizes this when considering the costs and benefits of mothering styles. Both forms have advantages and disadvantages but it is concluded that the styles adopted by mothers of different rank are adaptive because of the contrasting social pressures to which they are subjected by other group members. Arguments like this can only be speculative; they will become more convincing when larger samples of data are available.

The whole book is firmly based in modern evolutionary theory and those familiar with the parent-offspring conflict models of R. Trivers will find considerable interest in Altmann's analysis of weaning

and independence. It has been proposed that the behavioural conflict during weaning between a mother and her infant is a reflection of an underlying conflict of interest — the infant attempting to secure more maternal care than the mother has been selected to provide. With her extensive field data behind her, Altmann helps to shift this argument from a theoretical to a practical plane and concludes that "parent-offspring genetic conflict of interest may arise infrequently as a relevant variable in many real-life situations".

In conclusion this book offers much to all those interested in social behaviour. Its holistic approach is attractive since it clearly acknowledges the undeniable complexity of primate social relationships but it does, however, leave one anxious for the day when sample sizes will be larger and selection pressures better understood. □

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Magnetic resonance for biochemists and biophysicists

Karl-Erik Falk

ESR and NMR of Paramagnetic Species in Biological and Related Systems. Edited by I. Bertini and R.S. Drago. Pp.434. (Reidel: 1980.) Dfl.95, \$50.

THIS volume comprises the papers given at a NATO Advanced Study Institute meeting held in June 1979, the subject of which was the magnetic resonance of paramagnetic species of biological importance. Twenty-four expert authors have contributed chapters covering various aspects of this large and important area of research.

The first part of the book is devoted to NMR and to the effects of paramagnetism

on the NMR parameters, with chapters on the basic theory of relaxation and pulse techniques, paramagnetic effects in NMR, and a number of short reviews of current research. The applications for obtaining structural and dynamic information on various paramagnetic biological systems, ranging from purified small proteins to cell suspensions, are also discussed. The section on ESR contains a thorough consideration of theoretical aspects, focused on the 3d metal ions, followed by a discussion of the various classes of metalloproteins and, especially, the role of the metal ions. Throughout the book emphasis is put on the coupling between the unpaired electron and the nucleus, and about half of the volume deals with the complex effects of this interaction.

As a whole, the book is well balanced. NMR and ESR are given equal prominence, with theoretical descriptions, educational examples and reviews of current research results. Several of the

chapters contain useful tables concerning, for example, the interpretation and description of the abbreviations used in multipulse NMR, and the network of equations and assumptions that form the basis for understanding paramagnetic relaxation. The discussion of the various metalloproteins clearly reveals the importance and applicability of magnetic resonance techniques in unravelling the biological role of the metal ions.

The collected writings are generally interesting and well prepared, although there is some duplication of material, perhaps inevitable in a multiauthor publication. Overall, then, a good book which will be of value to biochemists and biophysicists with an interest in the biological application of magnetic resonance. □

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- AVRIEL, M. (ed.) *Advances in Geometric Programming. Mathematical Concepts and Methods in Science and Engineering*. 21. Pp. x + 460. ISBN 0-306-40381-1. (Plenum: 1980.) \$39.50.
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- HELD, A. (ed.) *General Relativity and Gravitation. One Hundred Years After the Birth of Albert Einstein*. Volume 1. Pp. xix + 598. ISBN 0-306-40265-3. (v.1.) (Plenum: 1980.) \$49.75.
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